Synthetic studies on the norditerpenoid alkaloid methyllycaconitine from delphinium, a potent nicotinic acetylcholine receptor antagonist

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SYNTHETIC STUDIES ON THE NORDITERPENOID ALKALOID METHYLLYCACONITINE FROM *DELPHINIUM*, A POTENT NICOTINIC ACETYLCHOLINE RECEPTOR ANTAGONIST

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Abstract

In this thesis, the exploration of the roles of the unusual acyl group of the toxic norditerpenoid alkaloid MLA, a highly potent novel competitive nicotinic acetylcholine receptor antagonist which is uniquely selective for the α-bungarotoxin binding site in both insects and vertebrates, is described. Chapter 1 is a review of the literature relating to the history, occurrence, and biological action of MLA and other norditerpenoid alkaloids.

The optimization of the extraction of crude norditerpenoid alkaloids from the seeds of Garden Hybrid *Delphinium* is described in Chapter 2. Vacuum liquid chromatography led to the isolation of pure MLA and delpheline. Characterization of these natural alkaloids and of semi-synthetic derivatives, including lycoctonine, obtained from the saponification of MLA, and esters of lycoctonine, using spectral techniques, helped to establish unambiguously stereochemistry of the methyl substituent on the succinimide of MLA.

Chapter 3 describes the development of sensitive high performance liquid chromatography assay for the routine monitoring of MLA levels in plant extracts and semi-synthetic alkaloid samples, prior to biological testing.

In Chapter 4, the design of a series of small molecule analogues of MLA, and the preparation of acylated cholines and homocholines, from isatoic anhydride, are described. The incorporation of the succinimide ring found in MLA into these anthranilate esters, by fusion with methylsuccinic anhydride, is also described.

Future work is to include synthesis of further esters of substituted anthranilic acid and esterification of lycoctonine with alternative aromatic and aliphatic acid chlorides, in order to explore further the pharmacophore of MLA.

The norditerpenoid alkaloid natural products and the esters of substituted anthranilic acid have been assayed for nicotinic potency in competition binding assays to rat brain membranes and for inhibitory effects on binding of ^{3}H-α-bungarotoxin to cockroach head homogenate, in order to explore the structural features of MLA required for activity.

Dedication

This thesis is dedicated to my family, namely, my parents Philip and Anne Coates, my sister Elizabeth, her husband Ian, their daughter Olivia, my brother Robert, my brother David, and his wife Stacy, all who have given me continual encouragement and love throughout the years of my research.

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Abbreviations

°C	degrees Celsius
AIBN	α,α'-azobisisobutyronitrile
Ac	acetyl
^{i}Am	isoamyl / isopentyl
aq	aqueous
bp	boiling point
Bu	butyl
^{i}Bu	isobutyl
^{n}BuLi	^{n}butyl
^{t}Bu	*tert*-butyl / tertiary butyl
Bz	benzoyl
CI	chemical ionisation
COSY	correlated spectroscopy
CSA	(+)-10-camphorsulfonic acid
2D	two dimensional
DCC	*N,N*-dicyclohexylcarbodiimide
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DIBAL	diisobutylaluminium hydride
DMAP	4-(*N,N*-dimethylamino)pyridine
DME	1,2-dimethoxyethane (glyme)
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
EI	electron impact
Et	ethyl
FAB	fast atom bombardment
GC	gas chromatography
h	hours
HPLC	high performance liquid chromatography
Hz	Hertz
IC_{50}	50% inhibitory concentration
IR	infrared
J	coupling constant

K_i	inhibitory concentration
LDA	lithium isopropylamide
LHMDS	lithium 1,1,1,3,3,3-hexamethyldisilazide
M	molar
$M^{+\cdot}$	molecular radical cation
MCPBA	*meta*-chloroperbenzoic acid
Me	methyl
MH^+	protonated molecular ion
min	minutes
MOM	methoxymethyl
mp	melting point
Ms	mesyl /methanesulfonyl
MS	mass spectroscopy
MW	relative molecular weight
m/z	mass by charge
^{n}	normal (or straight-chain)
NBS	*N*-bromosuccinimide
NMR	nuclear magnetic resonance
nOe	nuclear Overhauser effect
PCC	pyridinium chlorochromate
Ph	phenyl
ppm	parts per million
Pr	propyl
^{i}Pr	isopropyl
pTSA	*para*-toluenesulfonic acid
rt	room temperature
THF	tetrahydrofuran
THP	tetrahydropyranyl
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
$^v/_v$	volume by volume
$^w/_v$	weight by volume

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 METHYLLYCACONITINE

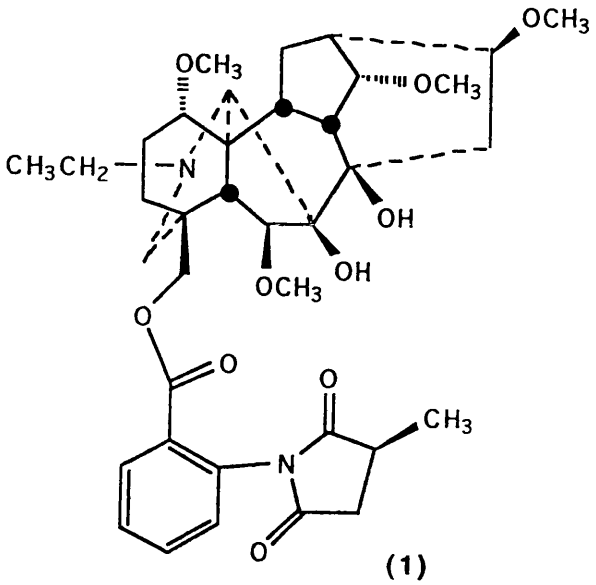
1.1.1 Structure and Isolation

Methyllycaconitine (MLA) (1) was first isolated by Manske, in 1938, from the aerial parts of *Delphinium brownii* Rydberg. Hydrolysis of the amorphous base gave the norditerpenoid lycoctonine (2), methylsuccinic acid and anthranilic acid, thus establishing MLA as the (-)-*N*-(*o*-carboxyphenyl)methylsuccinimide ester of lycoctonine (2) (Goodson, 1943). In 1943, Goodson reported MLA to be the major alkaloid of *Delphinium elatum* L. seeds and, since that date, there have been at least twenty eight reports of its occurrence in other *Delphinium* species and also in *Consolida ambigua* and *Inula royaleana*.

1.1.2 Biological Activity

MLA (1) has been identified as an important toxic principal in *Delphinium* (Nambi Aiyar *et al.*, 1979) and found to be responsible for producing pronounced insecticidal activity (Jennings *et al.*, 1986).

The insecticidal activity results from potent antagonism of insect nicotinic cholinergic receptors. In an assay which measured the inhibition of [^{3}H]-propionyl-α-bungarotoxin binding in housefly heads a K_{inh} value of less than 0.5nM was obtained (Jennings *et al.*, 1986 and 1987). In a cockroach motorneurone system (Sattelle *et al.*, 1989) MLA's blocking action was found to be independent of the membrane potential so suggesting competitive antagonism. It is likely that the synaptic blockade at neuronal nicotinic cholinergic synapses is due to postsynaptic actions of MLA, but a presynaptic role cannot be ruled out. In addition, irreversible blocking at the recognition site remains a possibility (Sattelle *et al.*, 1989).



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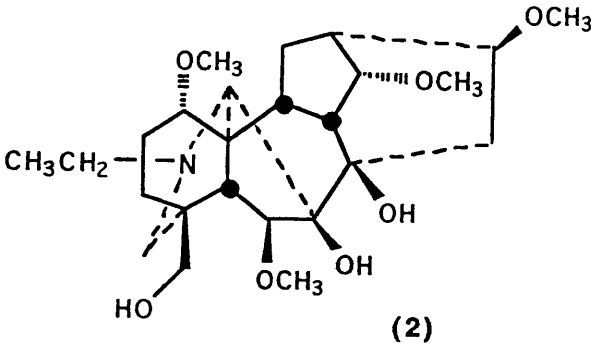

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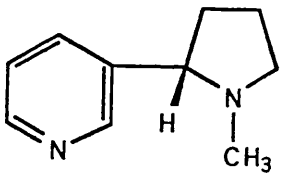

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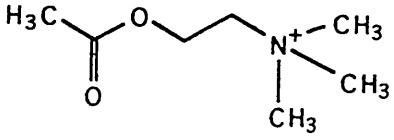

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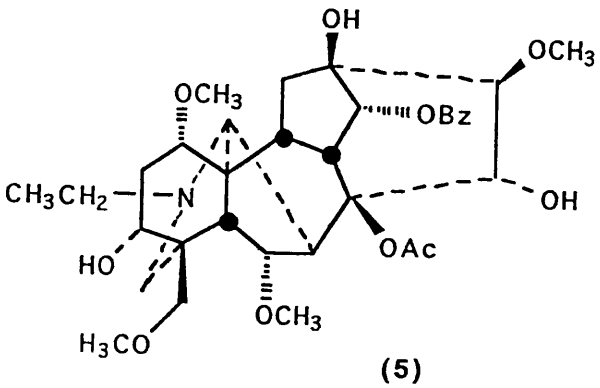

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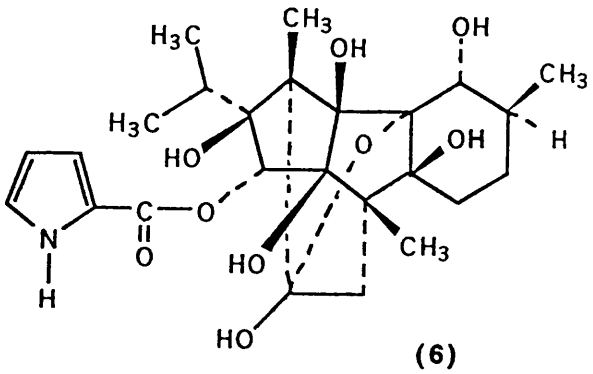

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Prominent among the symptoms of *Delphinium* poisoning in mammals is paralysis, similar to that produced by curare, suggesting that a primary site of action is the neuromuscular junction. As expected, MLA (1) showed significant nicotinic blocking action in an isolated rat phrenic nerve system, and no muscarinic activity using a guinea-pig ileum preparation. The EC_{50} for this effect is 2.3μM. It is interesting that the blockade was partially reversed by eserine, an acetylcholine esterase inhibitor (Nambi Aiyar *et al.*, 1979).
In another experiment, complete muscle paralysis (at 10^{-7}M MLA) was achieved after only 5 minutes. Thus, it appears that MLA is a potent nondepolarizing neuromuscular blocking agent acting competitively at nAChR.

The anti-cholinergic activity of MLA (1) at the rat muscle receptor has been found to be many times less potent than that at insect neuronal nicotinic receptors. This indicates significant differences between acetylcholine receptors in insects and vertebrates. MLA has also provided evidence to support the hypothesis that there are two distinct types of vertebrate nAChR: peripheral and neuronal (Macallan *et al.*, 1988). In the light of further experiments using MLA, another subdivision can be made. Within brain there appear to be both α-bungarotoxin binding sites and (*S*)-(-)-nicotine (3) binding sites. MLA potently inhibits ^{125}I-α-bungarotoxin binding in both a rat brain preparation and a locust ganglion preparation, but is a less effective inhibitor of (-)-[^{3}H]-nicotine binding (Wonnacott *et al.*, 1993). Moreover, MLA displays a much higher affinity for locust neuronal α-bungarotoxin binding sites than the equivalent site in the rat. In a different experiment, carried out using rat, MLA has been found to show markedly higher affinity for neuronal α-bungarotoxin binding sites than for muscle α-bungarotoxin binding sites. MLA is the first low molecular weight ligand to be able to make such a discrimination and is a useful preferential probe for this subclass of receptor (Ward *et al.*, 1990 and Alkondon *et al.*, 1992). MLA is, in fact, the most potent non-proteinaceous, low molecular weight toxin reported at this site. It has been found to be 10,000-fold more active as an antagonist than the agonist nicotine (3) and 25 times more so than the antagonist α-bungarotoxin (Jennings *et al.*, 1986).

Lycoctonine (2), the parent primary (neopentyl-like) alcohol, exhibits little neuromuscular blocking action, suggesting that MLA's aromatic ester functional group may have significance in the structure-activity profile (Manners *et al.*, 1993). It has been proposed by Ward *et al.* (1990) that MLA's high cholinergic nicotinic activity is associated with the *N*-benzoylsuccinimide side-chain and, in particular, with the carbonyl oxygen of the ester linkage; this electronegative centre (a feature of nicotinic ligands according to current models) probably bonds with key residues on the protein receptor ion-channel complex. MLA (1), whose lycoctonine portion is thought, during binding, to face out into the solvent surrounding the binding site, incorporates an acylated homocholine-like (3-aminopropan-1-ol) unit [see acetylcholine (4)] and so, the tertiary nitrogen is thought to interact electrostatically with the recognition site, in a similar way to the quaternary ammonium ion of ACh. One of the main criteria for nicotinic cholinergic activity is the distance between the nitrogen atom and the electronegative atom of the affinity ligand and it seems that the disulfide between cysteines 192-193 of the α subunit of the receptor complex (which are absent in β, γ and δ subunits) may interact with these key moieties (Kao and Karlin, 1986). It is believed that the precise spatial relationship between the key residues may account for MLA's marked preference for neuronal α-bungarotoxin binding sites compared with other nicotinic sites (Ward *et al.*, 1990). Therefore, MLA and its semi-synthetic analogues, and in particular the acylated anthranilic acid moiety in the supposed MLA pharmacophore, are clearly at the centre of an active field of research.

The high degree of structural rigidity in the skeleton (See Section 1.4.4.2) appears to effect the pharmacology of this and other related neurotoxic diterpenoid alkaloids (Codding, 1982).

1.2 AIMS OF THESE STUDIES

The aims of these studies are to explore the roles of the aromatic ester functional group of the toxic norditerpenoid alkaloid MLA (1). Examination of the seeds of Garden Hybrid *Delphinium* for MLA and related alkaloids, and synthesis of small molecule analogues, designed to contain the unusual substituted anthranilate moiety found in MLA, promise to assist in establishing the pharmacophore of this highly potent novel competitive nAChR antagonist.

As part of the structure-activity relationship (SAR) studies, phytochemical tasks must include optimization of the extraction of crude alkaloids from the seeds of Garden Hybrid *Delphinium*, assessment of various purification techniques, identification of any pure alkaloids isolated using spectral techniques and subsequent verification of the structure of MLA, including assignment of stereochemistry of the methyl substituent on the succinimide. Saponification of MLA (1) to give lycoctonine (2) offers the potential of endorsing the significance of the acyl group and of obtaining semi-synthetic esters of lycoctonine to examine the structural features required for activity.

A method of assessing the purity of norditerpenoid alkaloids obtained by extraction is a necessity, in order to validate the biological data obtained in competition binding assays to rat brain membranes and cockroach head homogenate and thus understand the trends in nicotinic potency for synthetic substituted anthranilate esters and the complex tertiary amine natural products.

Also, we have designed a series of small molecule analogues of MLA to incorporate succinimide ring moieties similar to the one observed in MLA. It is proposed to prepare these esters of substituted anthranilic acid from acyclic, monocyclic and bicyclic *N*-substituted ethanolamines and propanolamines.

1.3 DELPHINIUM AND ACONITUM

1.3.1 Plant Classification and Nomenclature

Kingdom	Plantae
Phylum	Spermatophyta
Division	Angiospermae
Class	Dicotyledoneae
Subclass	Archichlamydeae
Order	Ranunculales
Family	Ranunculaceae
Genus	*Delphinium* and *Aconitum*
Species	*Delphinium elatum* and *Aconitum carmichaeli*

Species may be further subdivided into varieties.

The scheme which is universally adopted for every organism and therefore, every plant is the *binomial system*, whereby, each species is given a name which consists of two Latin words. The first name denotes the genus and is always written with a capital letter, while the second (specific) name denotes the species. Then, these two are followed by the abbreviated name of the botanist(s) who were first responsible for describing the species under that particular name. So, e. g. the initial L. in *Delphinium elatum* L. is for Linnaeus.

The Ranunculaceae family contains 59 genera, including *Aconitum*, *Delphinium* and *Consolida* and incorporates many of the best known wild flowers in Britain, including Marsh Marigold, Buttercups, Clematis, Anemone, Hellebores and Adonis.

1.3.2 General

Delphinium are commonly tall, blue-flowered plants found in many European gardens as ornamentals and also in Siberia, Southeast Africa and the United States. It is believed that the first *Delphinium* to be named and recorded is *Delphinium staphisagria* some 2000 years ago. The name *delphinium* apparently originates from the similarity of the unopened flower bud to a little dolphin. In addition, together with *Consolida*, *Delphiniums* are commonly known as Larkspur, earning this name because the flowers are spurred, resembling a lark's claw. Red, white and yellow examples of these commonly grown perennial or biennial plants are found and estimations at the number of known species in the genus vary from 250 (Evans, 1989) to in excess of 500 (Edwards, 1981).

Aconitum are also usually blue or purple, but yellow examples are known with cultivated forms showing deeper colours. The genus comprises of approximately 300 known species (Evans, 1989), which are found in temperate regions of the Northern Hemisphere and from Western Europe to India and Japan.

The name *aconitum* and the old common name, wolfsbane, may originate from the Greek for javelin and wolf slayer, respectively, in recognition of the use of these plants as arrow tip poisons. The modern day common name, monkshood, comes from the cowl shape of the upper sepal.

1.3.3 Hybridization

There is little doubt that early hybridization involving *D. elatum* has led to the hardy perennial garden hybrid *Delphinium* cultivated extensively throughout the gardens of the world today. However, there is considerable confusion as to

the identity of any other species in this multiple cross, as the records of early botanists are now thought to be somewhat unreliable. *D. grandiflorum*, *D. formosum*, *D. exaltatum* and *D. chinense* may have been involved in its ancestry. Although the genetic make-up of plants is still not fully understood, it is now possible to obtain new hybrids with desirable characteristics, such as, colour, resistance to disease and frost, hardiness and variation in alkaloid content, found in both parents. Most hardy perennial *Delphinium* are agreed to be self-fertile, so in order deliberately to cross different cultivars, it is necessary to prevent the seed-bearing parent from producing pollen (with which it would otherwise fertilize itself) before exposure to the pollen of the other parent. Under these experimental conditions the natural mutation rate can be greatly increased, rather than relying on the wind, insects and bees, resulting in a cultivar with a different number of chromosomes in the plant cell nucleus and qualities inherited from both parents.

Similarly, there are approximately 100 popular hybrids of *Aconitum* known in science with *A. napellus* most commonly found to be involved in the ancestry of these crosses (Griffiths, 1994).

1.3.4 Toxicity

Native *Delphinium* species are considered to be responsible for more cattle deaths in North America than any other poisonous plant (Keeler, 1975 and Manners *et al.*, 1993). They are evidently palatable to livestock and account for an annual loss of up to 12% of cattle-stock worth millions of dollars. Other reported examples of accidental poisonings involve sheep, goats, horses and, most significantly, children. The signs of mammalian toxicosis from these plants are: loss of muscular control, salivation, trembling, rapid and weak respiration and heart action, bloating and ultimately respiratory paralysis. There is now no doubt that the diterpenoid alkaloids [including MLA (1)] present in the plant are responsible for the toxic properties described.

Aconitum preparations have long been used as weapon poisons in big-game hunting (a use listed in the Merck Index for *Aconitum ferox*) and also to dispose of criminals in medieval trials. *Aconitum* has been reported as mistaken for horse-radish so resulting in accidental poisonings. As a consequence of its extremely high toxicity towards mammals, of all the diterpenoid alkaloids, most attention has been given to aconitine (5) (Tomlinson *et al.*, 1993). Aconitine, found only in *Aconitum*, is readily absorbed through the skin and this is accompanied by itching and burning sensations along with an unpleasant numbness. Aconitine (5) is known to exhibit its neurotoxicity as a consequence of interacting with excitable membranes so as to hold open sodium-channels characterized by batrachotoxin. A fatal dose of aconitine (2-5mg in humans) results in cardiovascular impairment and respiratory inhibition.

1.3.5 Insecticidal Activity

As early as AD 77, as described by Pliny the Elder, crushed *Delphinium* seed preparations were used as a topical treatment for "vermin of the head and other parts of the body". More recently, there have been reports of the use of Stavesacre oil (the oil of *D. staphisagria* L.) as a pediculicide (anti-lice) effective against head and body lice infestations and bed-bugs. Apparently, a similar preparation was also a standard issue in the British Army at the time of the battle of Waterloo (Benn and Jacyno, 1983).

These plant extracts are now known to produce mortality in a broad spectrum of insects and mites, and this potent insecticidal action has now been attributed to certain of the alkaloids present. It is notable that there has been recent renewed interest in natural plant products for insect-control as a result of problems with resistance, toxicity and expense with synthetic compounds. Over 2000 species of plants are known to possess some insecticidal activity, many of which act on the nervous system of insects. In the future, therefore, these *Delphinium* alkaloids may lead to an insecticide of commercial importance.

MLA (1) is a competitive nAChR antagonist, and as an insecticide it may be compared with nicotine (3), an nAChR agonist. As early as 1763 nicotine [from *Nicotiana* (Solanaceae)], in the from of a tea prepared from tobacco, was recommended for the destruction of aphids. The use of other pyridine-based alkaloids grew to five million pounds by the mid-1900's, but attempts to use nicotine as a model for new insecticides have been commercially unsuccessful. Nicotine kills the insects by acting at nAChR (Jennings *et al.*, 1987). (Both nicotinic agonists and antagonists can possess insecticidal properties. The former overexcites nAChR, and the latter causes blockade of neuronal transmission and, therefore, paralysis.) Another nicotinoid with insecticidal properties is ryanodine (6), a complex ester of pyrrole-1-carboxylic acid, found in *Ryania speciosa* (Flacourtiaceae). This diterpenoid alkaloid causes cessation of feeding and flaccid paralysis in insects (European cornborer) by inhibiting the binding of calcium to muscle protein, and retards circulation by vascular constriction. One of the first insecticides used by man was aconite (Frohne and Pfänder, 1983), which consists of the dried roots of *Aconitum napellus* and contains aconitine-type diterpenoid alkaloids as well as aconitic acid and abundant starch.

1.3.6 Folk Medicine

The medicinal use of *Delphinium* extracts spans many centuries (as described in an extensive review by Benn and Jacyno, 1983). Different cultures have used *Delphinium* preparations for a wide variety of ailments, such as in sedatives, emetics, anthelmintics, analgesic balms and muscle relaxants and also in treating jaundice, dropsy, postencephalic parkinsonism, spinal arachnoiditis and disorders of the spleen. It is believed that the diterpenoid alkaloids present in these herbal remedies give rise to the therapeutic effects observed and so these traditional medicines may have implications for the design of modern-day drugs, in particular, with respect to the treatment of stroke, epilepsy and other forms of neurodegeneration.

The uses of *Aconitum* preparations, in oriental medicine over the centuries, have varied from treatments for fracture, bruises, dorsalgia waist, joint sprain, and traumatic injury to the treatment of rheumatic and rheumatoid arthritis, gout, gastroenteritis, diarrhoea, dyspepsia, abdominal pain, diabetes, and even toothache and throat infections. These herbal remedies have also been used as febrifuges, cardiotonics, sedatives, antipyretics and anodynes. Extracts of *Aconitum* have also been used for hypertension, neuralgia, lumbago, tuberculosis and diabetes along with controlling debility after fever and for curing hysteria. Anti-cancer use has also been described, but remains unproven, as do the reported aphrodisiac qualities!

Curariform activity and the associated structure activity relationships of other individual norditerpenoid alkaloids have been reviewed by Benn and Jacyno (1983) and by the Russian researchers, Mashkovsky and Churyukanov (1986).

1.4 DITERPENOID ALKALOIDS

1.4.1 Nature and Definition of an Alkaloid

The term "alkaloid" is not easy to define due to the lack of a clear-cut boundary between alkaloids and naturally occurring complex amines. A typical alkaloid (alkali-like) is derived from plant sources, it is basic, it contains one or more nitrogen atoms (usually in a heterocyclic ring) and it usually has a marked physiological action on man or other animals. An alkaloid, taken in the broadest sense, can have a nitrogen which is primary, secondary, tertiary or quaternary and can exist as a salt, amine or *N*-oxide. Alkaloids are principally of interest to humans because of their varied medicinal properties. They occur mainly in flowering plants and number about 10,000 structures, possessing an array of structural features.

1.4.2 Definition of a Diterpenoid Alkaloid

Classification of alkaloids is usually based on the type of ring system present (e. g. pyrrolidine, piperidine etc.) and on the biosynthetic origin from one or other of the protein amino acids. A rigid hierarchal classification is difficult to devise as some fit more than one category and others are best defined by their botanical occurrences.

MLA (1) and related alkaloids are classified as terpenoid alkaloids. They fall into the category of pseudo-alkaloids, as opposed to being regarded as true alkaloids, that is, the carbon skeleton is not derived from amino acids. Terpenoids can be conveniently classified according to the number of isoprene units present, as hemiterpene, monoterpene, sesquiterpene, diterpene, sesterpene and steroidal alkaloids (1-6 units, C_5-C_{30} carbon skeletons). It follows, therefore that the diterpenoid alkaloids of interest in this study are

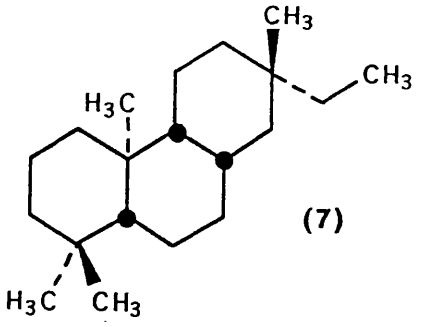
thought to be derived from C_{20} compounds, such as pimarane (7) or kaurane (8) (Pelletier and Mody, 1981). Diterpenoid alkaloids can be described as a group of highly oxygenated and complex natural compounds which may be divided into three broad categories, C_{20}-diterpenoid alkaloids, C_{19}-diterpenoid (norditerpenoid, i. e. diterpenoid lacking one carbon atom) alkaloids and miscellaneous diterpenoid alkaloids, such as *Erythroleum* (Leguminosae), *Ryania* (Flacourtiaceae) and *Taxus* (Taxaceae) alkaloids in which the nitrogenous moiety is linked to a non-nitrogenous diterpenoid skeleton *via* an ester linkage [well known examples of miscellaneous diterpenoid alkaloids include cassaidine (9), ryanodine (6) and taxol].

Diterpenoid alkaloids of the first two types occur mainly in the family Ranunculaceae (*Aconitum*, *Delphinium*, *Consolida*, *Thalictrum* and *Atragene*), but have also been obtained from *Garrya* (Garryaceae), *Inula* (Compositae), *Spiraea* (Rosaceae), *Icacina* (Icacinaceae), *Daphniphyllum* (Daphniphyllaceae) and *Anopterus* (Grossulariaceae).

An early observation was that the same diterpenoid alkaloid could occur in different species of both the *Aconitum* and *Delphinium* genera. Also, closely related plant species may be differentiated by the occurrence of similar, but different mixtures of alkaloids. In general, the biological role of alkaloids in plants is uncertain and they are classified as secondary metabolites. However, some of the diterpenoid alkaloids in *Aconitum* and *Delphinium* may function as insect feeding deterrents (Grina *et al.*, 1986 and Sattelle *et al.*, 1989).

1.4.3 Biosynthesis of C_{19}- and C_{20}-Diterpenoid Alkaloids

Biogenetically, C_{20}-diterpenoid alkaloids of the atisine-type (10) are possibly formed in nature from tetracyclic or pentacyclic diterpenes, such as kaurane (8). The aconane framework (11) may be derived formally from the atisine skeleton (12) by the loss of the exocyclic C(17) carbon, hence they can be termed the



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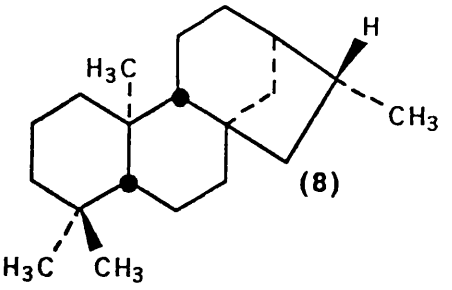

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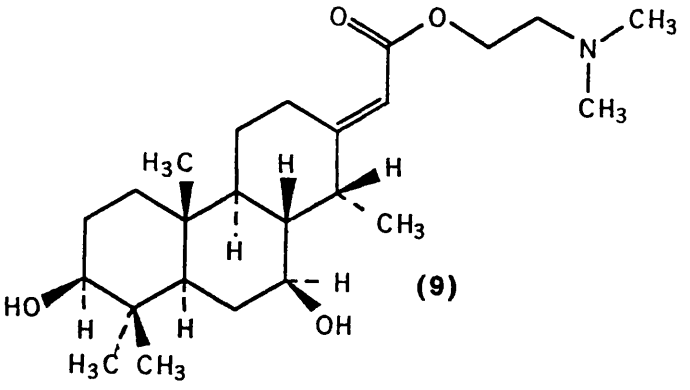

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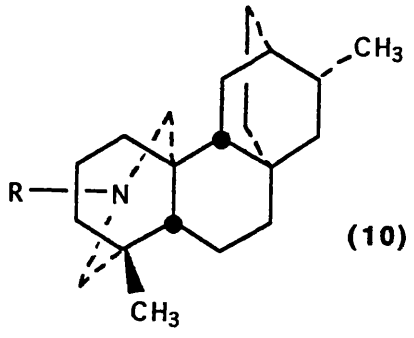

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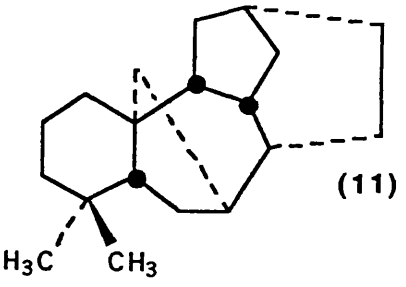

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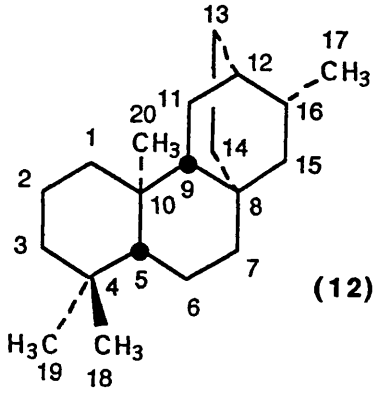

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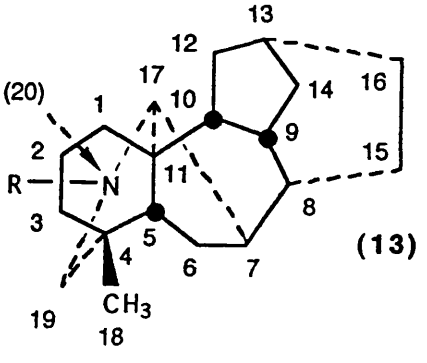

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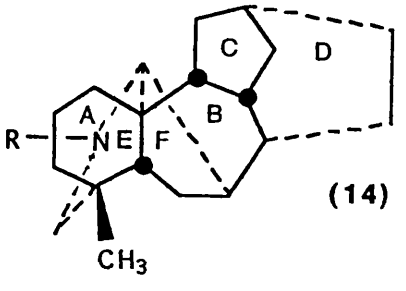

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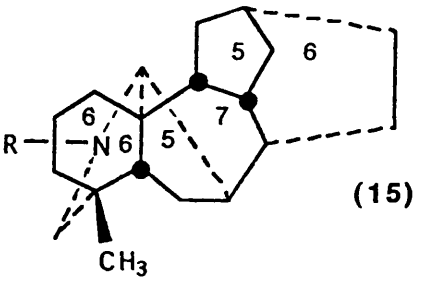


(15)

$C_{19}H_{28}$.NR Skeleton

17-nor-β-homo diterpenoid alkaloids. The C(9)-C(8) bond is believed to cleave or migrate to C(9)-C(14) in the atisane skeleton, thus generating a seven-membered ring B [see structures (13), (14) and (15) for ring nomenclature and numbering of the aconitane ring system]. During biosynthesis, the nitrogen is introduced formally by means of β-aminoethanol, methylamine or ethylamine linking to C(19) and C(20) in the C_{20}-diterpenoid skeleton and C(17) and C(19) in the C_{19}-diterpenoid skeleton to form a substituted piperidine ring (Pelletier and Mody, 1981).

1.4.4 C_{19}-Diterpenoid Alkaloids

1.4.4.1 Occurrence

Aconitum, *Consolida* and *Delphinium* have long been recognized as a rich source of C_{19}-diterpenoid alkaloids. To date, well over 300 such alkaloids have been isolated from *Aconitum*, *Consolida* and *Delphinium* (Liang *et al.*, 1990), the first of which was from *Delphinium staphisagria* L. by Brandes (1819) (Cook and Beath, 1952). In the last five years, more than one hundred and fifty new norditerpenoid alkaloids have been isolated and this trend continues.

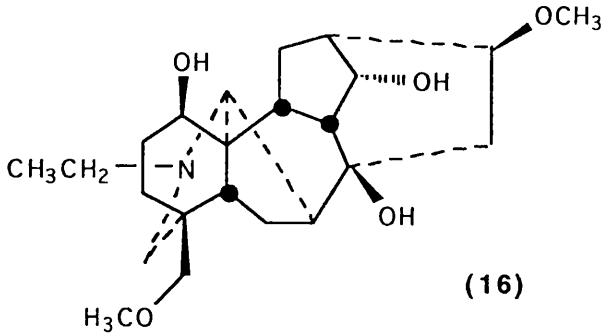
1.4.4.2 Structure

The C_{19}-diterpenoid alkaloids are polyhydric amino alcohols based on the formula $C_{19}H_{28}.NH$. The basic skeleton consists of two five-membered rings (C and F), three six-membered rings (A, D and E) and one seven membered ring (B) [structure (15)]. The E ring can be seen to be a piperidine ring. Most of the naturally occurring C_{19}-diterpenoid alkaloids have a tertiary nitrogen atom, one of whose substituents is a methyl or an ethyl group. The hexacyclic carbon skeleton is polyoxygenated with most examples bearing an oxygen function (acetoxyl, hydroxyl, methoxyl or ester group) at C(1), C(8), C(14) and C(16).

There are also some examples in which substitution with oxygen at C(3), C(7), C(9), C(10), C(13), C(18) and, more rarely, at the C(5) position are seen. Considering the C(1) position, there are no C_{19}-diterpenoid alkaloids with a β-methoxy group known in nature, but the β-configuration for hydroxyl groups in such bases as talatisidine (16) and delphirine (17) is known. The carbon skeleton of norditerpenoid alkaloids is reported to be extremely rigid such that ring D is a boat conformer, rings B and E are in distorted chairs, ring C is in an envelope with C(14) at the flap and ring F is in a half-chair. Ring A can be a boat or chair conformer (Codding, 1982) depending upon the substituent at C(1). Methoxy at C(1) favours a chair conformation whereas hydroxy at C(1) favours a boat which is stabilized by intramolecular hydrogen bonding to the nitrogen atom (Joshi and Pelletier, 1987). Structural elucidation of these highly complex alkaloids has been greatly facilitated by recent advances in spectroscopic techniques e. g. ^{1}H nmr, ^{13}C nmr (including 2-D techniques), high resolution mass spectroscopy and X-ray analysis and preeminent in this area is the research of Pelletier and co-workers (for recent reviews, see: Joshi and Pelletier, 1987, Pelletier *et al.*, 1977 and 1981a, Pelletier and Mody, 1979, Pelletier and Page, 1983 and 1986 and Pelletier *et al.*, 1984 and Pelletier and Joshi, 1991).

1.4.4.3 Types

The C_{19}-diterpenoid alkaloids can be broadly divided into four groups: (i) the lycoctonine-type which bears an oxygen function at C(7) on the aconane skeleton [structure (18)], e. g. lycoctonine (2), delcosine (19) and dictyocarpine (20); (ii) the aconitine-type which lacks an oxygen function at C(7) [structure (21)], e. g. aconitine (5), delphinine (22) and condelphine (23); (iii) the heteratisine-type which is characterized by a lactone moiety in ring C of the skeleton [structure (24)], e. g. heteratisine (25) and heterophylline (26); (iv) the pyrodelphinine-type in which a double bond is present between C(8) and C(15) [structure (27)], e. g. falaconitine (28) and mithaconitine (29).



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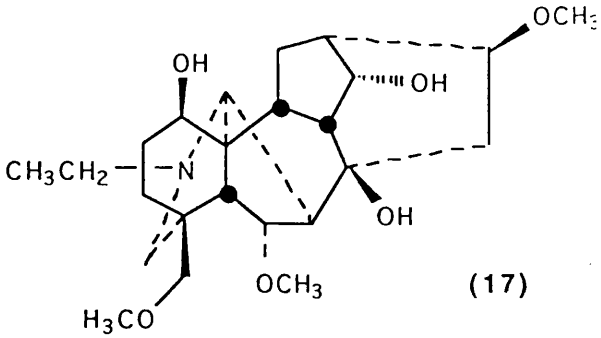

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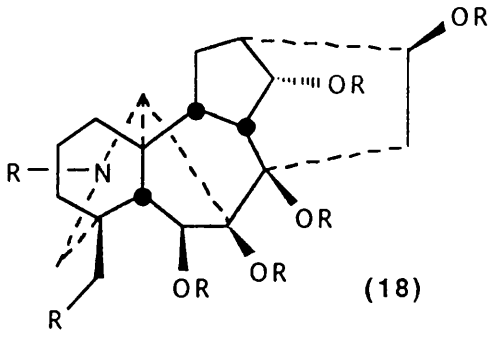

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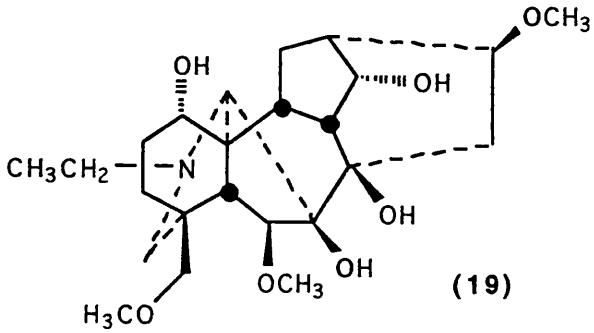

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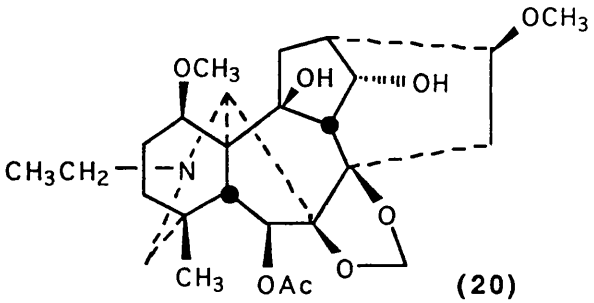

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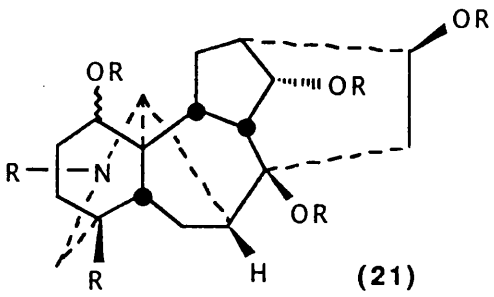

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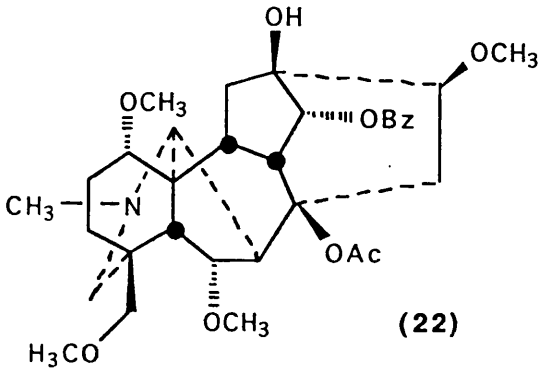

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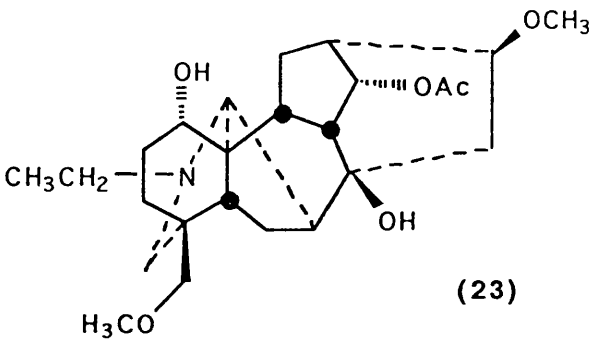

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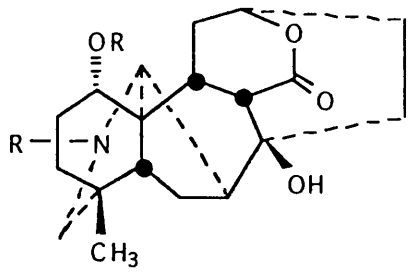

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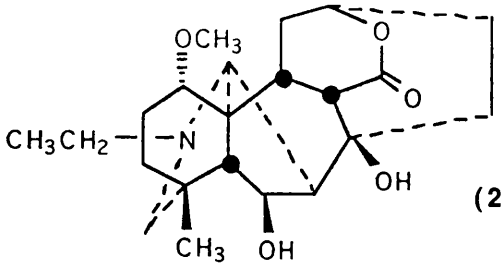

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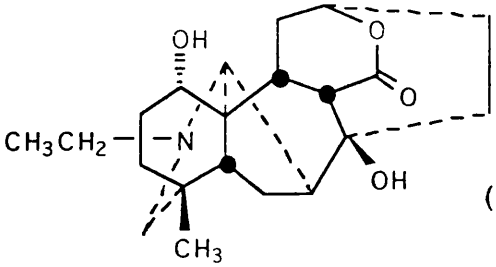

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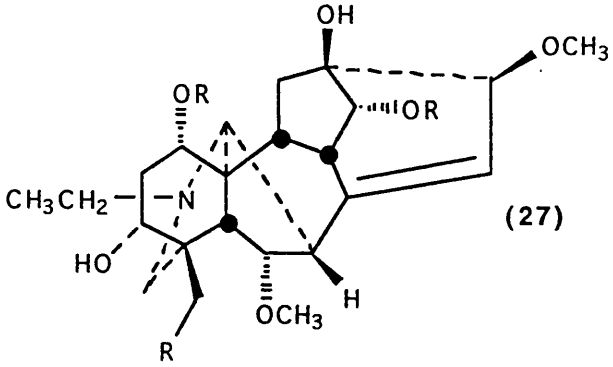

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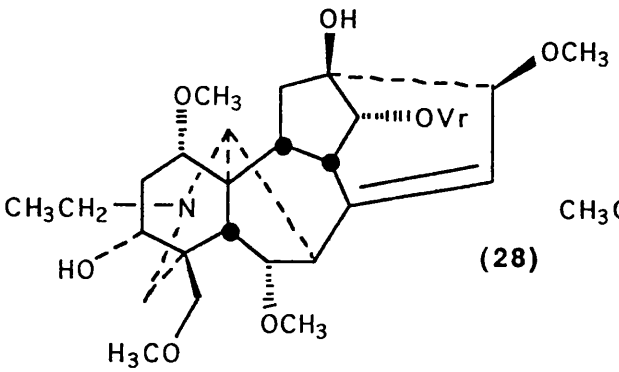

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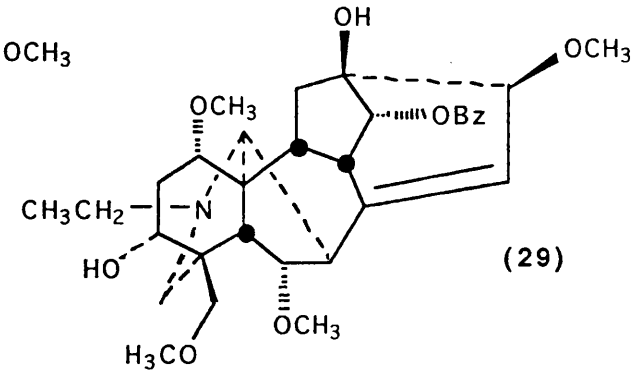

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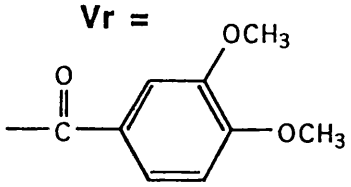


1.4.4.4 Nomenclature

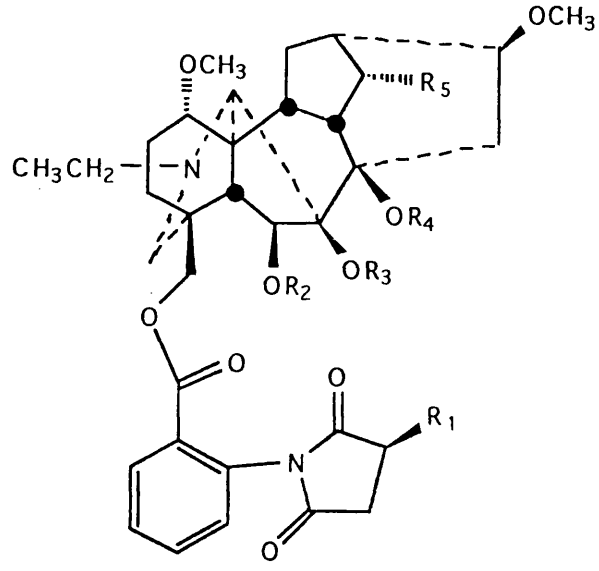
Generally, the name of an alkaloid ends in "-ine" and usually bears some relationship to the Latin botanical name of the plant from which it was first obtained. So, in the same way as atropine is from *Atropa belladonna* and aconitine (5) is from *Aconitum* then ajacine is from *Delphinium ajacis* L.

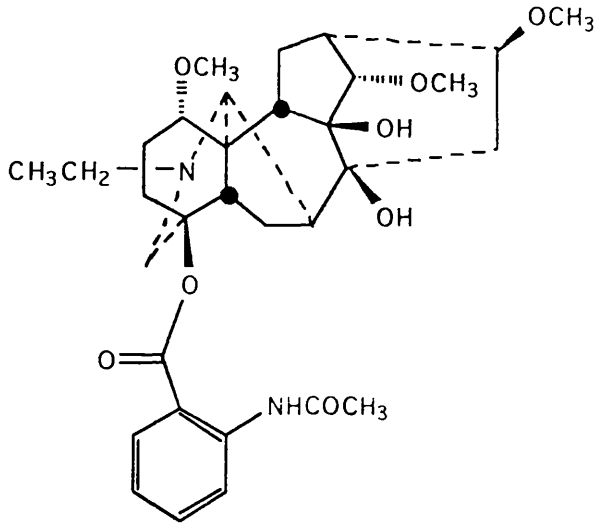
Systematic nomenclature of norditerpenoids is extremely complex, but in Chemical Abstracts they are based on the aconitane (13) skeleton, so for example, MLA (1) is 7,8-diol, 20-ethyl-1,6,14,16-tetramethoxy-4-{[(2-(3-methyl-2,5-dioxo-1-pyrolidinyl) benzoyl) oxy] methyl}-[1α,4(*S*),6β,14α,16β]aconitane. [21019-30-7].

Historically many of these alkaloids have been given more than one trivial name [e. g. MLA (1) can be known as delartine, delsemidine, or mellictine, lycoctonine (2) is otherwise called delsine or royaline, and another name for inuline (40) is anthranoyllycoctonine].

1.4.4.5 MLA and other aromatic esters of Lycoctonine-Type Diterpenoid Alkaloids

MLA (1) is the 2-(methylsuccinimido)benzoate ester of lycoctonine (2). Benn and colleagues have consistently drawn the stereochemistry of the methyl substituent on the succinimide as *S*, while most authors have left the chirality at this carbon centre as undefined. Other known C_{19}-diterpenoid alkaloids with *N*-phenylsuccinimide ester moieties are lycaconitine (30) (which differs from MLA only in that it lacks the methyl group on the succinimide ring), glaudelsine (31) [which has a hydroxyl group rather than methoxyl function at C(6)], elatine (32) (which has a methylenedioxy function between positions 7 and 8), anhweidelphinine (33) [which has N=C(19) and is an imine lacking the *N*-ethyl group], ajacusine (34), barbinine (35), elanine (36), nudicauline (37) and 14-deacetylnudicauline (38) which have benzyl, keto, 2-methylbutyryl, acetoxyl and hydroxyl functions respectively in position 14, in place of MLA's methoxyl group





Lappaconitine (39)

MLA (1)	$R_1 = R_2 = CH_3$, $R_3 = R_4 = H$, $R_5 = OCH_3$
Lycaconitine (30)	$R_1 = R_3 = R_4 = H$, $R_2 = CH_3$, $R_5 = OCH_3$
Glaudelsine (31)	$R_1 = CH_3$, $R_2 = R_3 = R_4 = H$, $R_5 = OCH_3$
Elatine (32)	$R_1 = R_2 = CH_3$, $R_3 + R_4 = CH_2$, $R_5 = OCH_3$
Anhweidelphinine (33)	$R_1 = R_2 = CH_3$, $R_3 = R_4 = H$, $R_5 = OCH_3$, C(17)H-N=C(19)H
Ajacusine (34)	$R_1 = R_2 = CH_3$, $R_3 = R_4 = H$, $R_5 = OBz$
Barbinine (35)	$R_1 = R_2 = CH_3$, $R_3 = R_4 = H$, $R_5 = OCOCH(CH_3)CH_2CH_3$
Elanine (36)	$R_1 = R_2 = CH_3$, $R_3 = R_4 = H$, $R_5 = =O$
Nudicauline (37)	$R_1 = R_2 = CH_3$, $R_3 = R_4 = H$, $R_5 = OAc$
14-Deacetylnudicauline (38)	$R_1 = R_2 = CH_3$, $R_3 = R_4 = H$, $R_5 = OH$

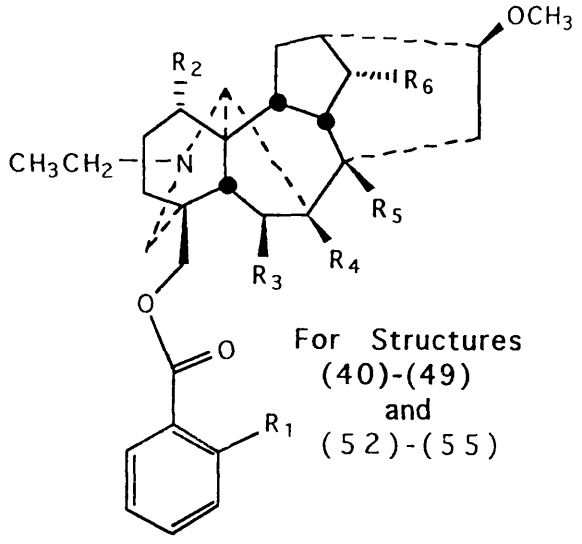
(for a recent review in *Natural Product Reports*, see: Yunusov, 1991). No alkaloids are known which possess the methylsuccinimido benzoate group at any position other than C(18), although lappaconitine (39) and related alkaloids, which lack C(18) have anthranoyl ester group at C(4).

Alkaloids with different aromatic acyl groups at C(18) are known (Yunusov, 1991). Anthranoyl lycoctonine (40), aconorine (41), ajacine (42), ajadine (43), andersonidine (44), delectine (45), delvestine (46), delvestidine (47), scaconitine (48), talassicumine A, B and C [(49), (50) and (51)] and their derivatives [(52), (53), (54), (55) and (56)] all have amine or acetylamine groups in place of the succinyl moiety. Septentrionine (57), septentriodine (58), andersonine (59) and delavaine A and B (60) are examples of alkaloids with the side-chains $NHCOCH_2CH_2COOCH_3$, $NHCOCH(CH_3)CH_2COOCH_3$ and $NHCOCH_2CH(CH_3)COOCH_3$. It is thought that the latter alkaloids may be artifacts from nudicauline and MLA, as a result of methanolysis of the cyclic imide with the primary alcohol solvent used in the separation procedures (Pelletier *et al.*, 1986a). Whereas, in the cases of avadharidine (61), delsemine (62) and bulleyanitine A (63) the succinimide ring is believed to have been opened by ammonia during isolation. Other alkaloids with similar side chains include glyanine A and B (64), puberaconitine (65) and puberaconitidine (66).

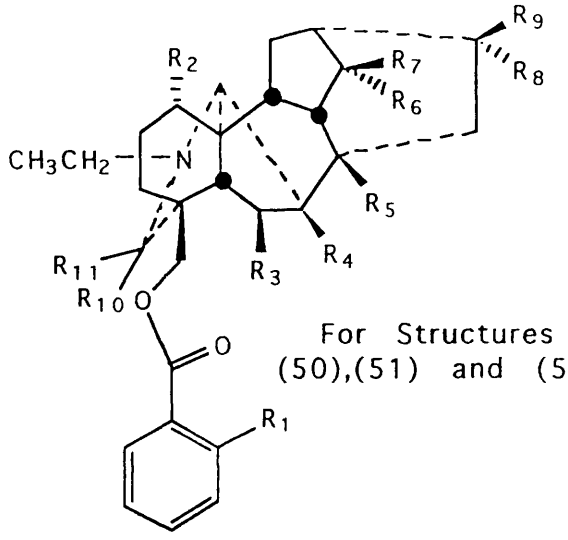
1.4.5 C_{20}-Diterpenoid Alkaloids

1.4.5.1 Occurrence

C_{20}-diterpenoid alkaloids have been isolated and characterized from *Delphinium*, *Aconitum*, *Consolida ambigua*, *Anopterus*, *Garrya*, *Spiraea* and *Thalictrum* species. These amino alcohols (alkamines) are relatively non-toxic and, in contrast to the highly oxygenated C_{19}-diterpenoid alkaloids, are not usually extensively oxygenated (Pelletier, 1992).

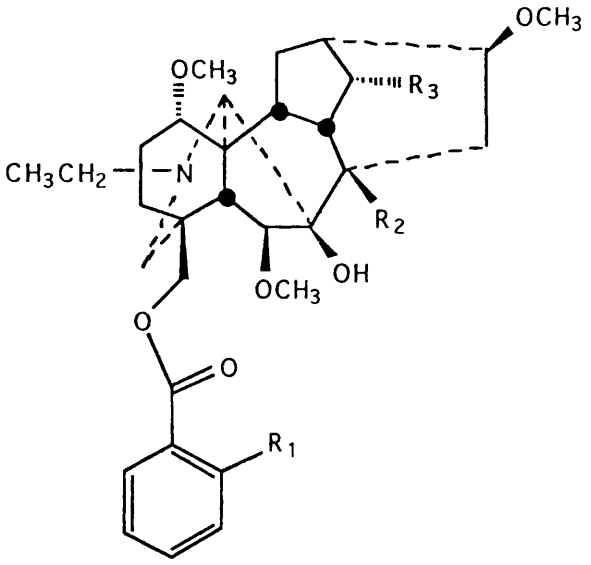


For Structures (40)-(49) and (52)-(55)



For Structures (50),(51) and (56)

Anthranoyllycoctonine /Inuline (40)	$R_1 = NH_2$, $R_2 = R_3 = R_6 = OCH_3$, $R_4 = R_5 = OH$
Aconorine (41)	$R_1 = NHAc$, $R_2 = OCH_3$, $R_3 = R_4 = H$, $R_5 = R_6 = OH$
Ajacine (42)	$R_1 = NHAc$, $R_2 = R_3 = R_6 = OCH_3$, $R_4 = R_5 = OH$
Ajadine (43)	$R_1 = NHAc$, $R_2 = R_3 = OCH_3$, $R_4 = R_5 = OH$, $R_6 = OAc$
Andersonidine (44) /14-Acetyldelectine	$R_1 = NH_2$, $R_2 = R_3 = OCH_3$, $R_4 = R_5 = OH$, $R_6 = OAc$
Delectine (45)	$R_1 = NH_2$, $R_2 = R_3 = OCH_3$, $R_4 = R_5 = R_6 = OH$
Delvestine (46)	$R_1 = NH_2$, $R_2 = R_4 = OH$, $R_3 = R_5 = R_6 = OCH_3$
Delvestidine (47)	$R_1 = NH_2$, $R_2 = R_3 = R_5 = R_6 = OCH_3$, $R_4 = OH$
Scaconitine (48)	$R_1 = NHAc$, $R_2 = R_6 = OCH_3$, $R_3 = R_4 = H$, $R_5 = OH$
Talassicumine A (49)	$R_1 = NHAc$, $R_2 = OCH_3$, $R_3 = R_4 = H$, $R_5 = OCH_2CH_3$, $R_6 = OH$
Talassicumine B (50)	$R_1 = NHAc$, $R_2 = R_3 = R_5 = R_8 = R_9 = R_{10} = R_{11} = H$, $R_4 = OCH_3$, R_6 & R_7 = O, plus C(8)=C(15)
Talassicumine C (51)	$R_1 = NHAc$, $R_2 = OCH_3$, $R_6 = OH$, plus C(16)=C(15), $R_3 = R_4 = R_5 = R_7 = R_8 = R_9 = R_{10} = R_{11} = H$
14-Deacatylajadine (52)	$R_1 = NHAc$, $R_2 = R_3 = OCH_3$, $R_4 = R_5 = R_6 = OH$
N-Acetyldelectine (53)	$R_1 = NHAc$, $R_2 = R_3 = OCH_3$, $R_4 = R_5 = OH$, $R_6 = OAc$
Isodelectine (54)	$R_1 = NH_2$, $R_2 = R_4 = R_5 = OH$, $R_3 = R_6 = OCH_3$
N-Deactylscaconitine (55)	$R_1 = NH_2$, $R_2 = R_6 = OCH_3$, $R_3 = R_4 = H$, $R_5 = OH$
(56)	$R_1 = NH_2$, $R_2 = R_3 = R_6 = R_9 = OCH_3$, $R_4 = R_5 = OH$, $R_8 = H$, R_{10} & R_{11} = O



Septentrionine (57) R_1 = $NHCOCH_2CH_2CO_2CH_3$, $R_2 = R_3 = OCH_3$

Septentriodine/ Cashmiradelphine (58) R_1 = $NHCOCH_2CH_2CO_2CH_3$, R_2 = OH, $R_3 = OCH_3$

Andersonine (59) R_1 = $NHCOCHCH_3CH_2CO_2CH_3$, $NHCOCH_2CHCH_3CO_2CH_3$, R_2 = OH, R_3 = OAc

Delavaine A+B (60) R_1 = $NHCOCHCH_3CH_2CO_2CH_3$, $NHCOCH_2CHCH_3CO_2CH_3$, R_2 = OH, $R_3 = OCH_3$

Avadharidine/ Awadcharidine (61) R_1 = $NHCOCH_2CH_2CONH_2$, R_2 = OH, $R_3 = OCH_3$

Delsemine (62) R_1 = $NHCOCHCH_3CH_2CONH_2$, $NHCOCH_2CHCH_3CONH_2$, R_2 = OH, $R_3 = OCH_3$

Bulleyanitine A (63) R_1 = C(17)-N=C(19), $NHCOCHCH_3CH_2CONH_2$, $NHCOCH_2CHCH_3CONH_2$, R_2 = OH, $R_3 = OCH_3$

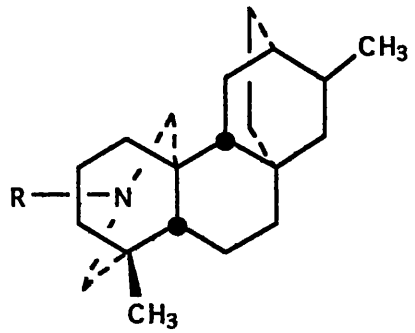
Gyalanine A+B (64) R_1 = $NHCOCHCH_3CH_2CO_2Et$, $NHCOCH_2CHCH_3CO_2Et$, R_2 = OH, $R_3 = OCH_3$

Puberaconitine/ *N*-(succinyl)anthranoyl lycoctonine (65) R_1 = $NHCOCH_2CH_2CO_2H$, R_2 = OH, $R_3 = OCH_3$

Puberaconitidine (66) R_1 = $NHCOCH_2CH_2CO_2H$, $R_2 = R_3 = OCH_3$

1.4.5.2 Types

The C_{20}-diterpenoid alkaloids can be broadly divided into four groups: (i) the atisine-type (10), which incorporates an *ent*-atisane nucleus (12) and does not obey the isoprene rule, e. g. atisines (67), denudatines (68), hetidines (69) and hetisines (70); (ii) the veatchine-type (71), which is modeled on an *ent*-kaurane nucleus (8), obeys the isoprene rule and has a five membered ring D, e. g. veatchines (72), napellines (73) and anopterines (74); (iii) the delnudine-type (75), e. g. delnudine [as (75)]; (iv) the bisditerpenoid-type (76), which is thought to be derived by attachment of two different molecules of C_{20}-diterpenoid alkaloids at C(16), e. g. staphisine [as (76)].



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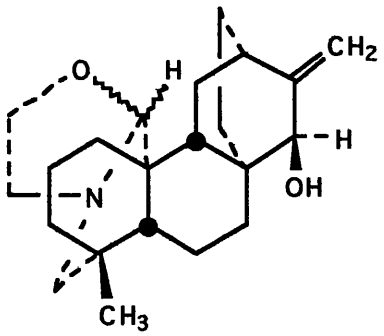

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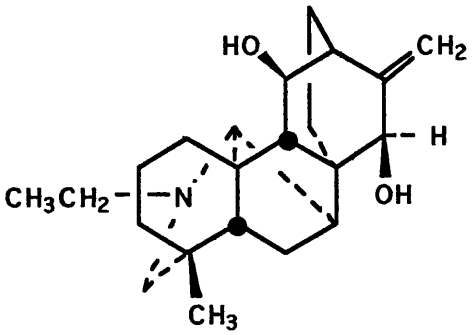

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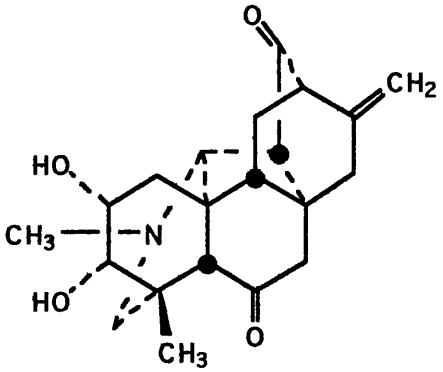

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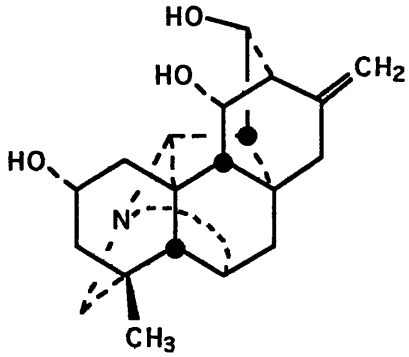

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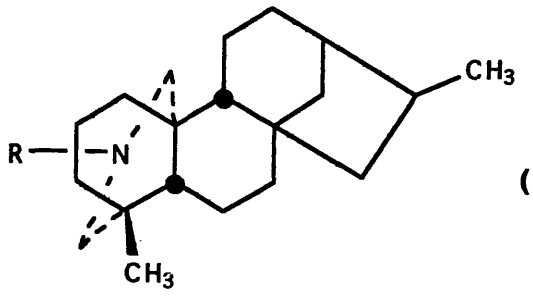

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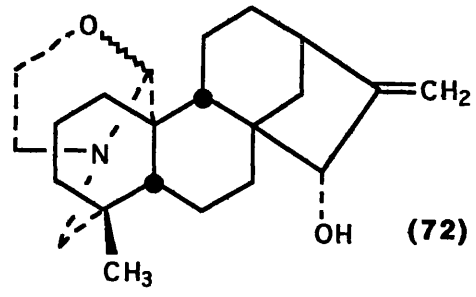

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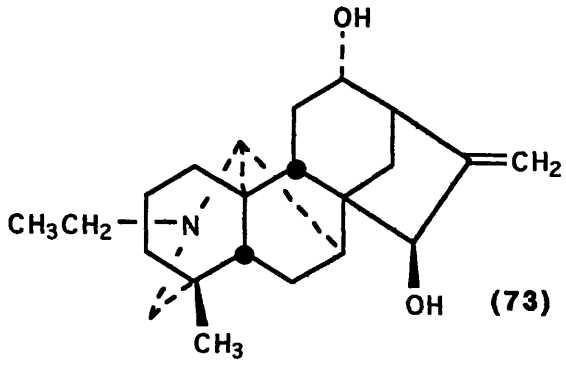

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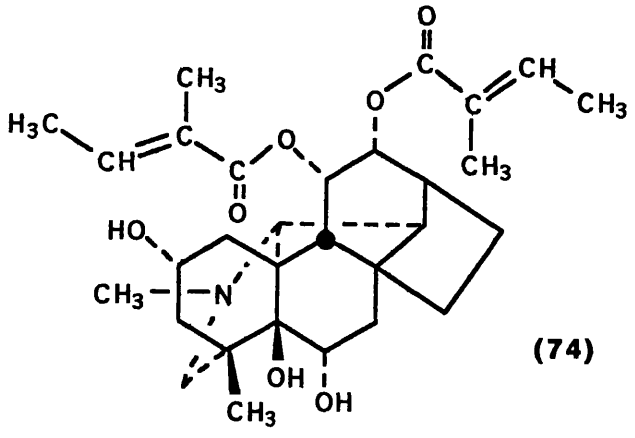

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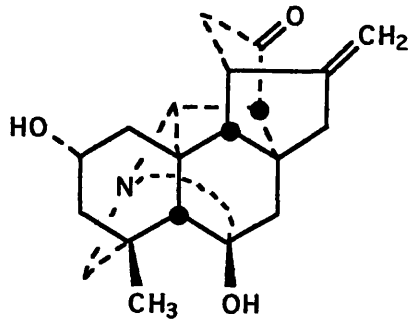

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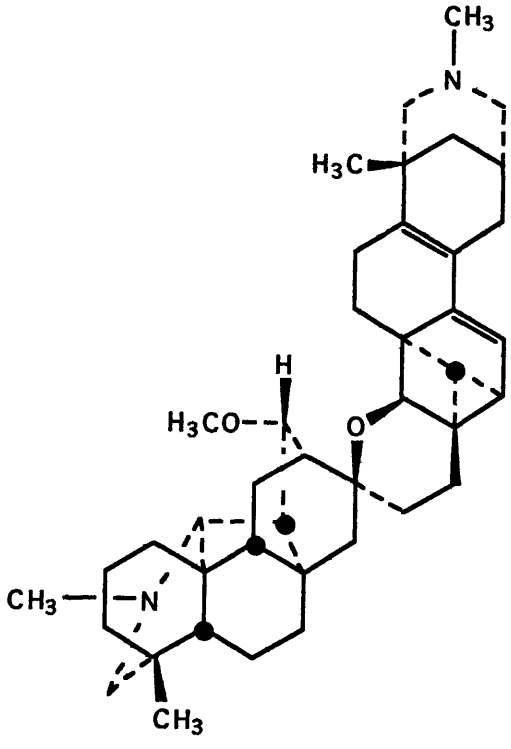
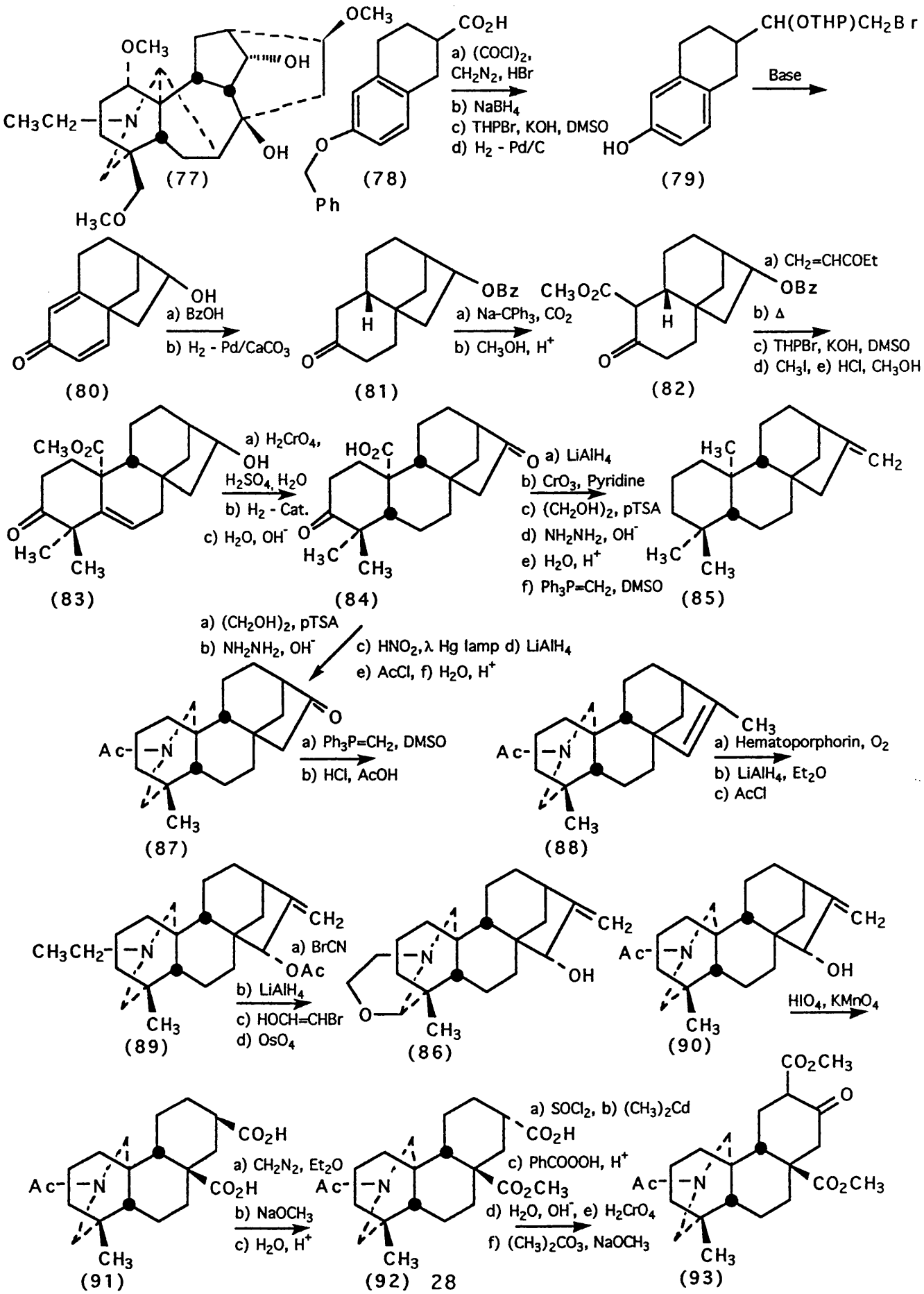


(76)

1.5 SYNTHETIC STUDIES OF C_{19}-DITERPENOID ALKALOIDS

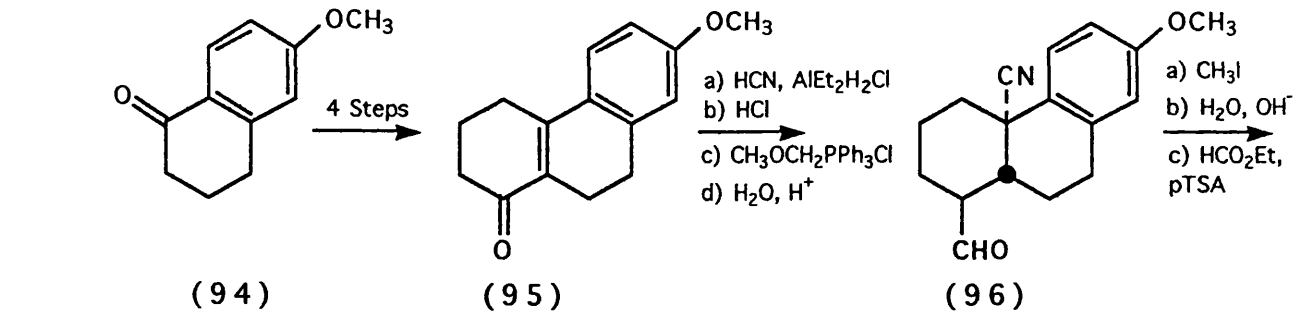
Throughout the 1970's and 1980's, Wiesner and co-workers at the University of New Brunswick made a phenomenal contribution to the area of diterpenoid chemistry. Publication of their achievements appeared as a great number of papers in prestigious refereed journals (e. g. Wiesner *et al.*, 1972, 1974a-d and 1977, Lee *et al.*, 1976, Atwal *et al.*, 1978, Tsai *et al.*, 1979 and Wiesner, 1979 and 1985) and in the form of numerous reviews (e. g. Pelletier and Page, 1978, 1981, 1982 and 1986, Pelletier and Mody, 1979 and Amiya and Bando, 1988). The research group successfully developed relatively simple, fully regio- and stereospecific methods for the synthesis of a number of C_{20}- and C_{19}-diterpenoid alkaloids. Their elegant synthetic strategies, in which the many functional groups materialized in the correct positions and configurations simultaneously with the construction of the diterpenoid skeleton, proved that these complex polycyclic polybridged and polysubstituted natural products can be synthesized. They frequently achieved their goals by using preliminary model studies to test the key reactions, resolve many significant problems and modify their synthetic approaches, before carrying out the total alkaloid syntheses. Whenever possible, they utilized well-known, reliable chemistry (Diels-Alder reactions, Birch reductions, photoadditions, Jones oxidations, Grignard reactions, Wolff-Kishner reduction, ketal protections, retro aldol cleavages and aldol condensations, to mention but a few) and for each alkaloid, a strategy similar in principle to earlier examples was employed, but frequently improving on their own methods or those of other research groups. A common feature to most of their work was an aziridine rearrangement step with synthetic routes often proceeding through partially aromatic, tricyclic, tetracyclic and pentacyclic intermediates. They began their synthetic investigations with relatively simple diterpenoid alkaloids [e. g. atisine (67), napelline (73) and talatisamine (77)] and progressed to more heavily substituted alkaloids [e. g. denudatine (68), aconitine (5) and delphinine (22)].

In 1964, Masamune (Masamune, 1964a-d) was investigating the synthesis of diterpenes and diterpenoid alkaloids. Masamune (1964a) reported the synthesis of a tetracyclic intermediate of the kaurane-type [structure (8)], achieved by converting carboxylic acid (78) into tetrahydropyranyl ether (79) in four steps. Phenol (79) was cyclized to give hydroxy dienone (80). Catalytic hydrogenation of the benzoate of (80) gave two isomeric tetrahydro compounds (81) and then carbomethoxylation and methylation of the isomer shown gave β-keto ester (82). Construction of ring A was achieved by addition of ethyl vinyl ketone, cyclization and exhaustive methylation (using protection), furnishing dimethyl compound (83). Keto acid (84) was obtained from (83) on oxidation to the diketone and reduction to the saturated derivative. Alternatively, kaurane compound (84) was prepared (Masamune, 1964b) by the degradation of the *Garrya* alkaloid, veatchine (72) in eight steps. The kaurane-type compound (84) can be converted into (-)-kaurene (85) in six steps. Thus, diterpenoid alkaloid was converted into diterpene and a direct correlation of the two groups of natural products was established. Intermediate (84) was also converted into the C_{20}-diterpenoid alkaloid, garryine (86), *via* intermediates keto amide (87), alkene (88) and allylic acetate (89) (Masamune, 1964c). Masamune (1964d) also converted veatchine (72) (possessing a [3.2.1]bicyclooctane CD ring structure) into atisine (67) (possessing a [2.2.2]bicyclooctane system). Compound (90) was prepared from veatchine azomethine acetate and then oxidized to afford dicarboxylic acid (91). Epimerization of dimethyl ester of (91) gave the *trans* compound, which on hydrolysis gave monoester carboxylic acid (92). Six steps afforded β-keto ester (93). Conversion of this into atisine (67) *via* the monomethyl ester carboxylic acid lacking the keto group, thus completed the formal synthesis of atisine.

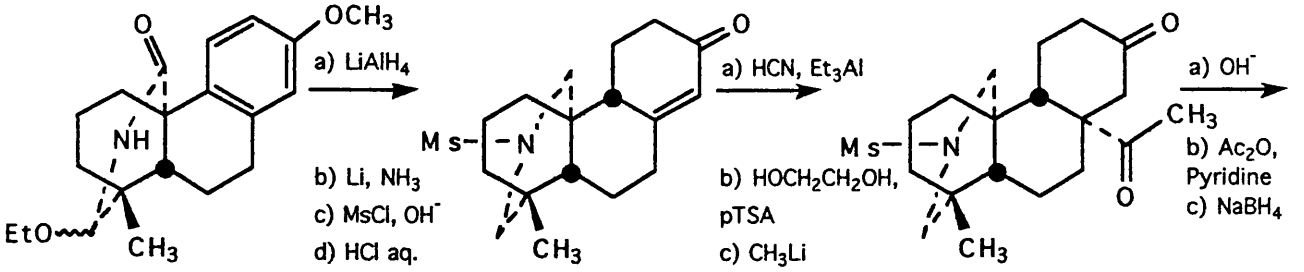

OCH3
OH
CH3CH2
N
OH
H3CO
(77)
OCH3
CO2H
O
Ph
(78)
a) (COCl)2,
CH2N2, HBr
b) NaBH4
c) THPBr, KOH, DMSO
d) H2 - Pd/C
CH(OTHP)CH2Br
HO
(79)
Base
OH
O
(80)
a) BzOH
b) H2 - Pd/CaCO3
OBz
H
O
(81)
a) Na-CPh3, CO2
b) CH3OH, H+
CH3O2C
OBz
H
O
(82)
a) CH2=CHCOEt
b) Δ
c) THPBr, KOH, DMSO
d) CH3I, e) HCl, CH3OH
CH3O2C
O
H3C
CH3
(83)
a) H2CrO4,
OH
H2SO4, H2O
b) H2 - Cat.
c) H2O, OH-
HO2C
O
H3C
CH3
(84)
a) LiAlH4
b) CrO3, Pyridine
c) (CH2OH)2, pTSA
d) NH2NH2, OH-
e) H2O, H+
f) Ph3P=CH2, DMSO
H3C
CH2
H3C
CH3
(85)
a) (CH2OH)2, pTSA
b) NH2NH2, OH-
c) HNO2, λ Hg lamp d) LiAlH4
e) AcCl, f) H2O, H+
Ac-
N
O
CH3
(87)
a) Ph3P=CH2, DMSO
b) HCl, AcOH
Ac-
N
CH3
CH3
(88)
a) Hematoporphorin, O2
b) LiAlH4, Et2O
c) AcCl
CH3CH2-
N
CH2
a) BrCN
OAc
CH3
(89)
b) LiAlH4
c) HOCH=CHBr
d) OsO4
CH2
N
OH
O
CH3
(86)
Ac-
N
CH2
OH
CH3
(90)
HIO4, KMnO4
Ac-
N
CO2H
a) CH2N2, Et2O
CO2H
b) NaOCH3
c) H2O, H+
CH3
(91)
a) SOCl2, b) (CH3)2Cd
CO2H
c) PhCOOOH, H+
Ac-
N
CO2CH3
d) H2O, OH-, e) H2CrO4
f) (CH3)2CO3, NaOCH3
CH3
(92)
CO2CH3
O
Ac-
N
CO2CH3
CH3
(93)

In the 1960's, Nagata and co-workers successfully synthesized three alkaloids in their racemic form. 6-Methoxy-1-tetralone (94) was converted into conjugated enone (95) by the Stork process in four steps and then hydrocyanation, epimerization [to give the *trans* compound with regards to the C(5)-C(10) bond], Wittig reaction and acid hydrolysis gave formyl compound (96) (Nagata *et al.*, 1967a). Stereoselective methylation, followed by alkaline hydrolysis and ethylation gave epimeric ethoxy lactams (97). Reduction of the keto function followed by Birch reduction to the corresponding dienol ether, mesylation and acid treatment gave conjugated ketone (98). In order to construct ring D, tetracycle (98) was hydrocyanated and the ketal protected nitrile was treated with methyl lithium. Acid hydrolysis gave the *trans* methyl ketone (99). Cyclization of (99) gave the hydroxy ketone derivative which was acetylated and stereoselectively reduced to give 15α alcohol (100). Mesylation, followed by hydrolysis and ring fragmentation with elimination gave vinyl derivative (101). Ketalization, hydroboration, oxidation and subsequent deketalization gave primary alcohol (102). Pentacyclic ketone (103) was obtained on mesylation and cyclization with K^{t}OBu. Wittig reaction of (103) afforded the *exo* methylene derivative, which on Birch reduction (deprotection) and *N*-acetylation gave (104). Bromination of exocyclic alkene (104) gave (105) which after epoxidation and treatment with zinc and ethanol gave allylic compound (106) and the 15β-hydroxy compound. Natural (106) has been transformed into atisine so this constituted the stereospecific total synthesis of *dl*-atisine (67) (Nagata *et al.*, 1963 and 1967a).

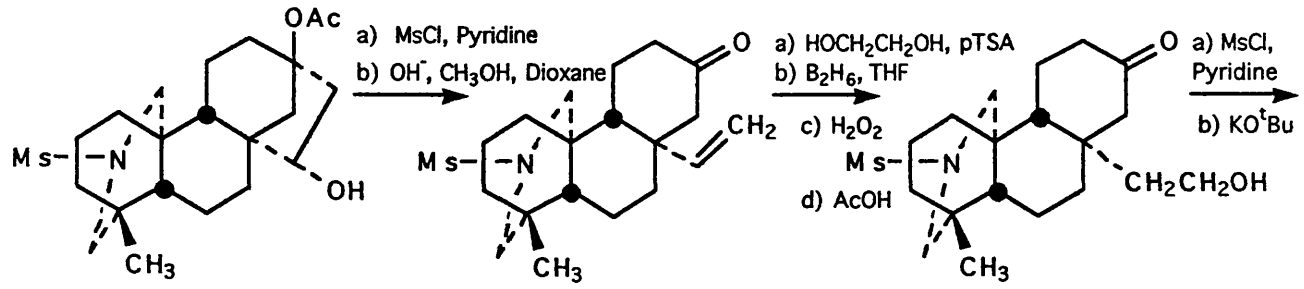
Nagata and co-workers also described the total synthesis of garryine (86) and veatchine (72) in racemic forms (Nagata *et al.*, 1964 and 1967b). Pentacyclic intermediate (107) was prepared from (95) in sixteen steps in a similar manner. It was necessary to convert the CD bridged system from the phyllocadene-type into the kaurene-type [see structure (8)] (opposite configuration). This was achieved by conversion of (107) into acetoxy alkene (108), then



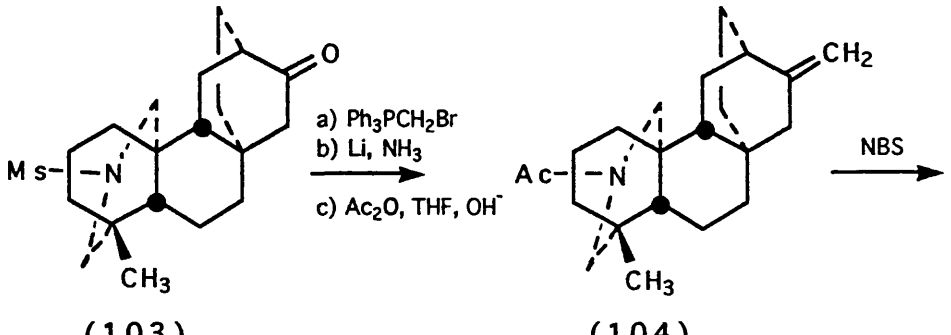
(94) (95) (96)



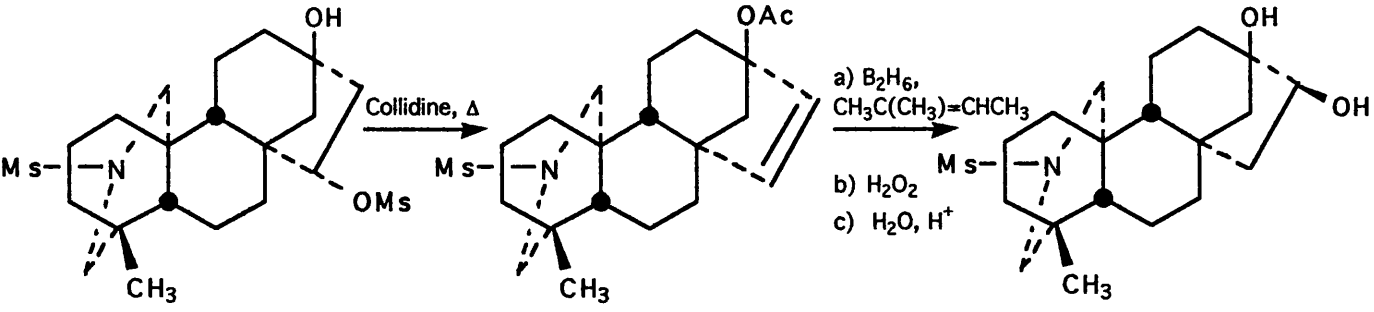
(97) (98) (99)



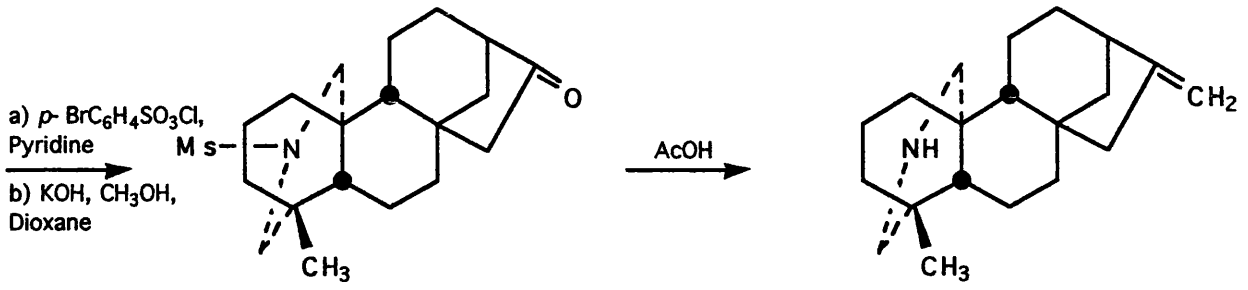
(100) (101) (102)



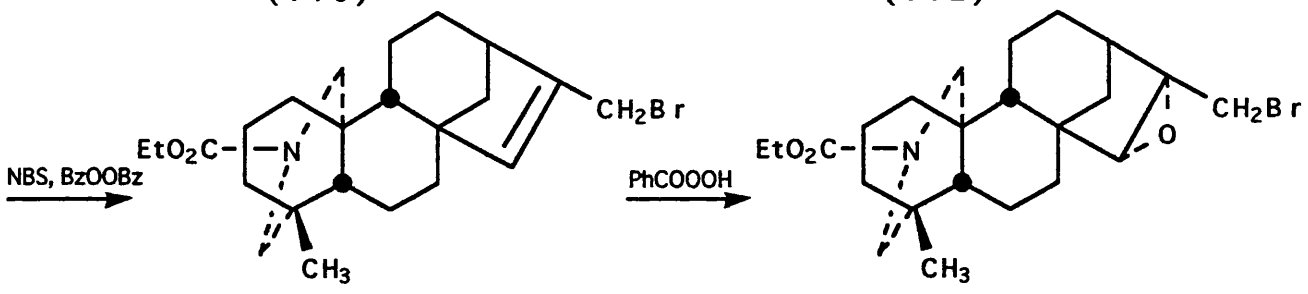
(103) (104) (105) (106)



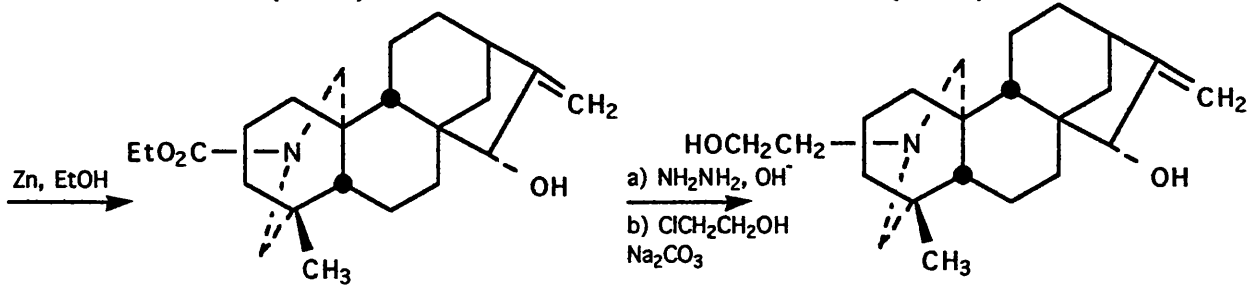
(107) (108) (109)



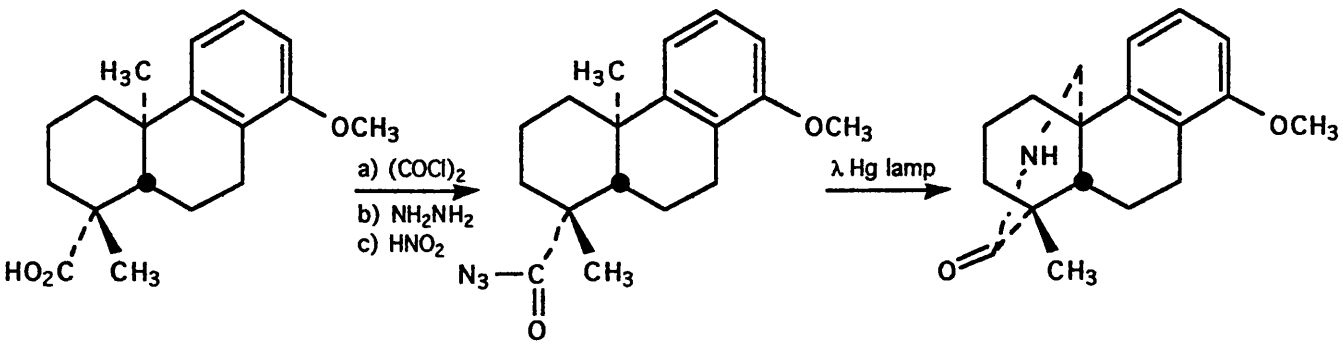
(110) (112)



(111) (113)



(114) (115)



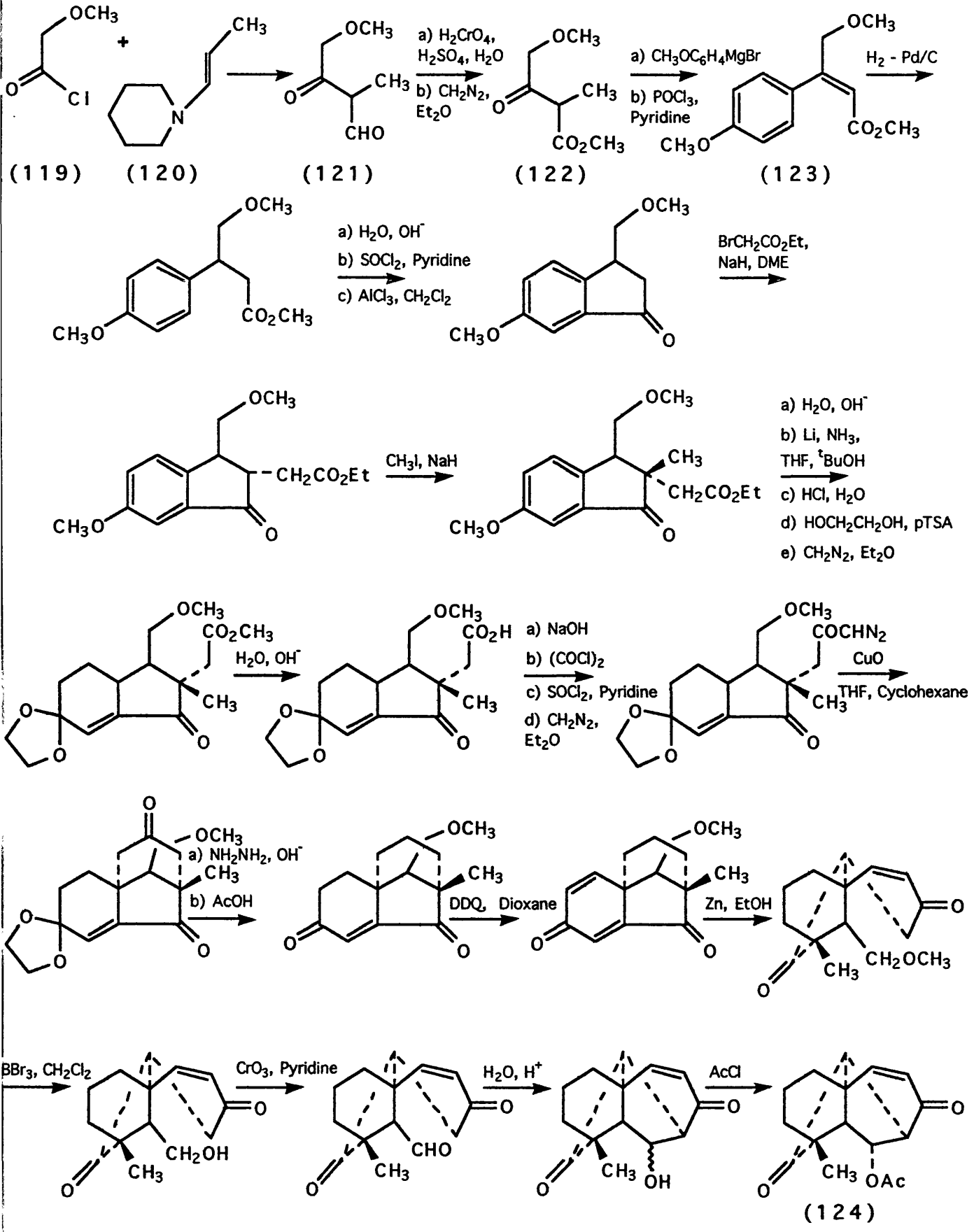
(116) (118) (117)

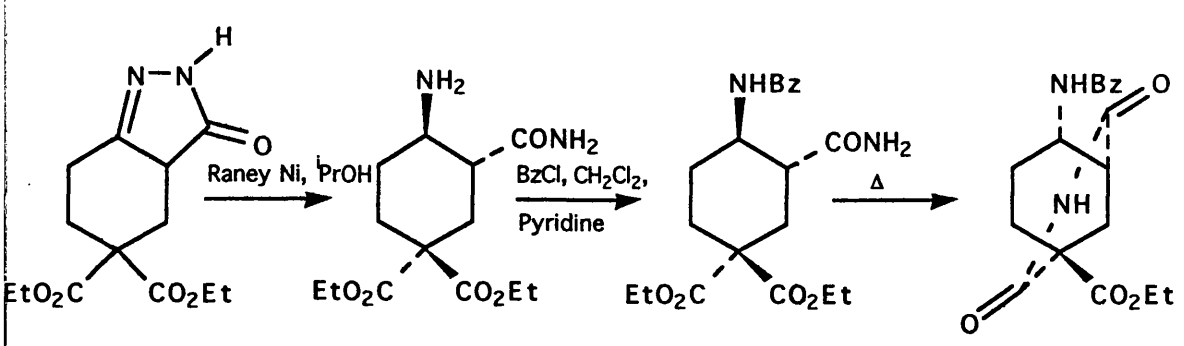
stereocontrolled hydroboration, oxidation and hydrolysis to give 1,2-diol (109). Selective sulfonation of the C(16) hydroxyl group prior to base-induced rearrangement gave ketone (110) with the appropriate bridge configuration. Allylic bromide (111) was obtained *via* demesylated secondary amine *exo* methylene derivative (112). Epoxidation of (111) gave epoxy bromide (113) which on treatment with zinc in ethanol gave allylic alcohol (114). Hydrolytic removal of the *N*-carbethoxy group followed by alkylation with ethylene chlorohydrin gave *dl*-dihydroveatchine (115). Natural (115) has been converted into garryine (86) and in a further two steps to veatchine (72) so this work constituted total syntheses of the racemic forms of these alkaloids.

In 1971, Mori *et al.* converted tricyclic acid (116) into tetracyclic lactam (117) which contains ring E of diterpenoid alkaloids such as garryine (86) and veatchine (72) (within Pelletier and Mody, 1981). This was achieved by conversion into acid chloride, hydrazide and azide (118) with photolysis finally yielding lactam (117).

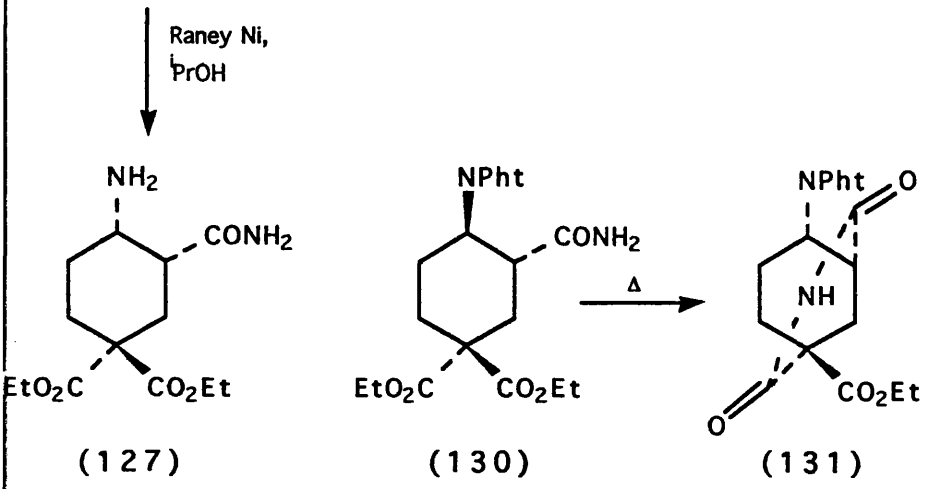
Cornforth and Penegelly reported (Cornforth, 1980 and Cornforth and Penegelly, 1982) numerous improbabilities and inconsistencies in the synthetic scheme devised by Chatterjee (1979) towards the synthesis of C_{19}-diterpenoid alkaloids. The former were unable to verify the first three stages (119 and 120 ->121->122->123) of the approximately twenty step route claimed by Chatterjee to the intermediate (124).

In 1980, Škarić and co-workers reported (Škarić *et al.*, 1980) their approach to the synthesis of 3-azabicyclo[3.3.1]nonane systems (AE bicycle) towards diterpenoid alkaloids. Reductive cleavage of indazole dicarboxylate (125) gave a mixture of aminocarbamoylcyclohexanes (126) and (127). To obtain pure stereoisomers it was necessary to prepare the *N*-benzoyl derivatives of the cyclohexylamines [one shown (128)]. Separated *trans*-benzamido

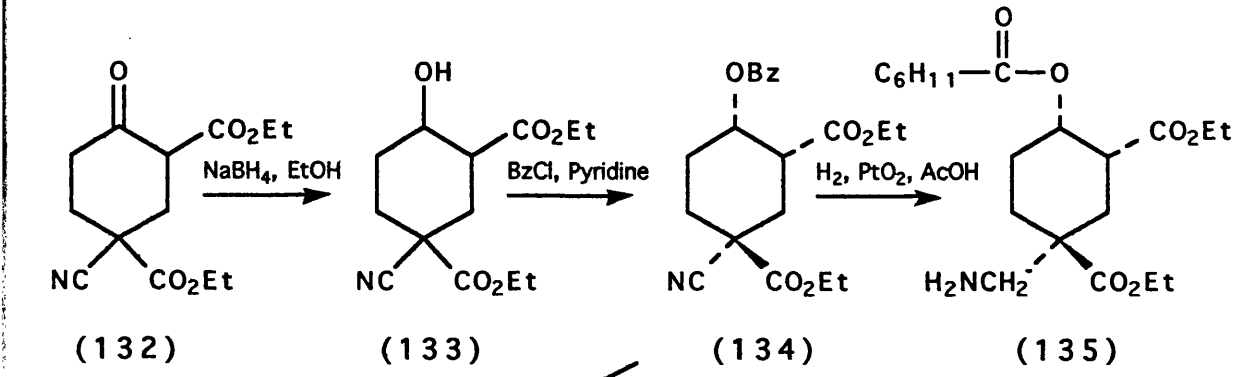

(119)
(120)
(121)
(122)
(123)
a) H_2CrO_4, H_2SO_4, H_2O
b) CH_2N_2, Et_2O
a) $CH_3OC_6H_4MgBr$
b) $POCl_3$, Pyridine
H_2 - Pd/C
a) H_2O, OH^-
b) $SOCl_2$, Pyridine
c) $AlCl_3$, CH_2Cl_2
$BrCH_2CO_2Et$, NaH, DME
CH_3I, NaH
a) H_2O, OH^-
b) Li, NH_3, THF, ^tBuOH
c) HCl, H_2O
d) $HOCH_2CH_2OH$, pTSA
e) CH_2N_2, Et_2O
H_2O, OH^-
a) NaOH
b) $(COCl)_2$
c) $SOCl_2$, Pyridine
d) CH_2N_2, Et_2O
CuO
THF, Cyclohexane
a) NH_2NH_2, OH^-
b) AcOH
DDQ, Dioxane
Zn, EtOH
BBr_3, CH_2Cl_2
CrO_3, Pyridine
H_2O, H^+
AcCl
(124)



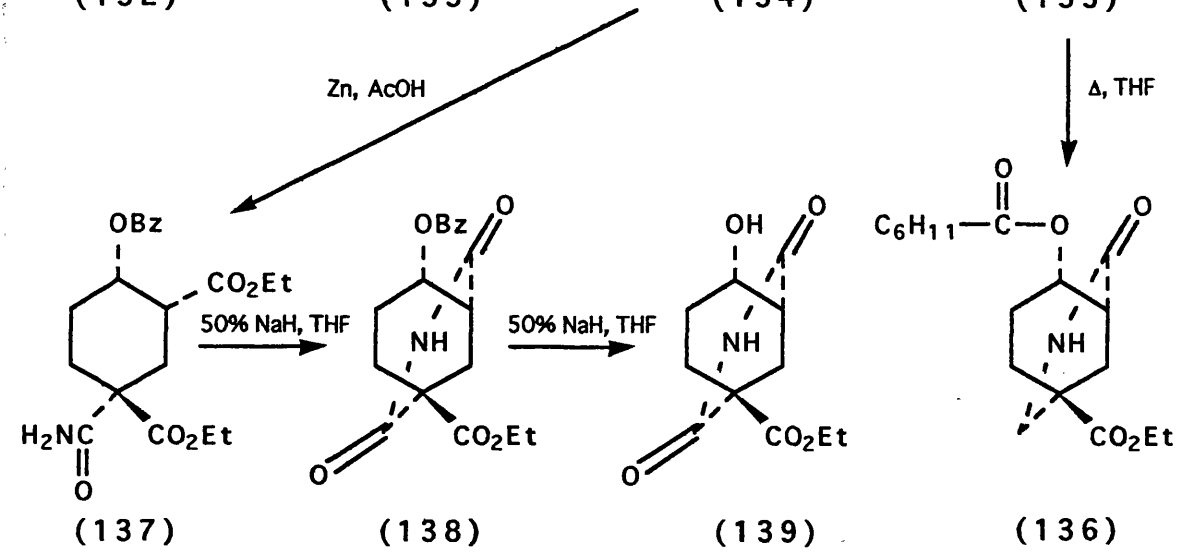
(125) (126) (128) (129)



(127) (130) (131)



(132) (133) (134) (135)

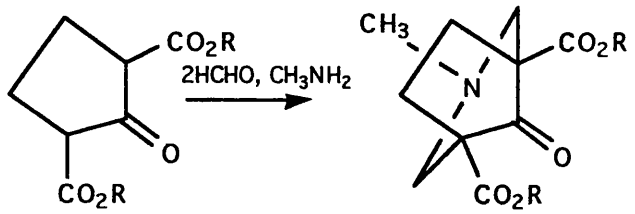


(137) (138) (139) (136)

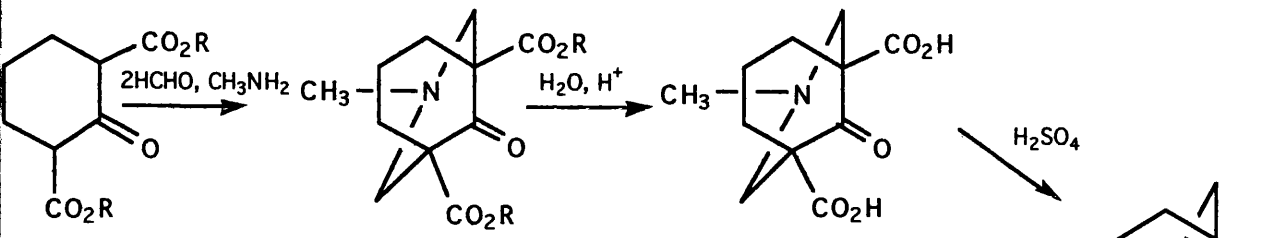
isomer (128) was converted by intramolecular cyclization, into bicycle (129), (*cis* stereochemistry). The proton at C(1) (norditerpenoid numbering) was established as equatorial (to the cyclohexane ring). A stereomeric mixture of (126) and (127), treated with phthalic anhydride gave *tertiary*-2-phthalimido compound (130) alone, which on cyclization gave (131), with the proton at C(1) again assigned as equatorial.

Škarić and co-workers also reported (Makarević and Škarić, 1988) the synthesis of bicyclic compounds with *O*-substituents in the C(1) (diterpenoid alkaloid numbering) position. Reduction of (132) gave a diastereomeric mixture of (133). Separation as the *O*-benzoyl derivatives [e. g. (134)] was achieved. Hydrogenation over PtO_2 in acetic acid gave (135), which was transformed into (136) on heating in THF. Equally successful was the hydration of (134) to give (137) (Makarević and Škarić, 1988). Intramolecular cyclization afforded (138) or in the presence of a larger amount of NaH, debenzoylation was facilitated, giving (139).

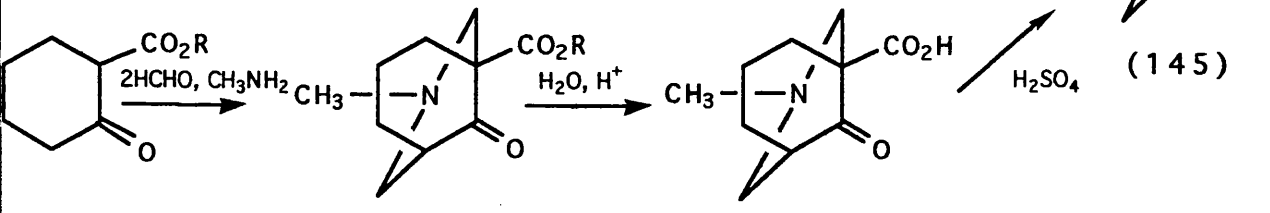
Shimizu *et al.* (1963) reported the use of the Mannich reaction in the synthesis of the AE-[3.3.1]bicyclic portion of the diterpenoid nucleus. This research group investigated five and six membered ring ketones with α carbon atoms bearing activating carboxylic groups. Thus, disubstituted ketones (140) and (141) were converted into (142) and (143), respectively, using two equivalents of formaldehyde and one equivalent of methylamine. 3-Azabicyclo[3.3.1]nonane (143) was then hydrolyzed to the corresponding diacid (144), which was decarboxylated to give (145). Next (146), having only one carboxyiic group at the α position to the keto group, was transformed into (145) *via* (147) and (148). Similarly, cyclohexanone (149), with a methyl group adjacent to the keto group was converted into (150), through (151) and (152). Finally, 2-aryl derivatives (153) (Ar = *p*-methoxyphenyl or *m*-methoxyphenyl), with no carboxylic group at the α-position, were converted into 2-aryl-6-methyl cyclohexanones (154) *via* the 6-formyl derivatives (155) (treatment with ethyl formate then catalytic hydrogenation). Azabicyclononane compounds (156) were then obtained as before.



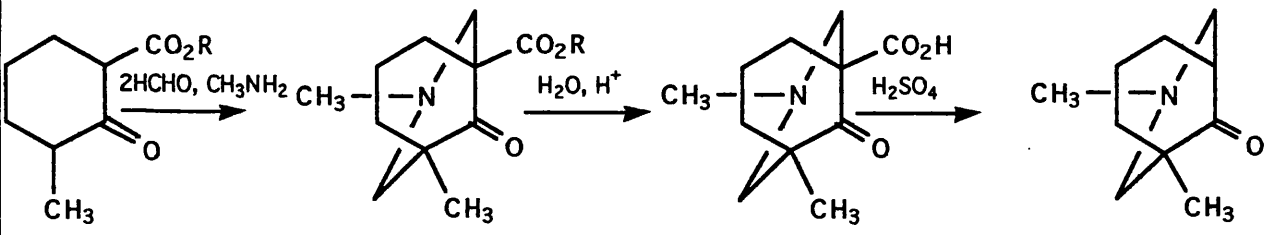
(140) (142)



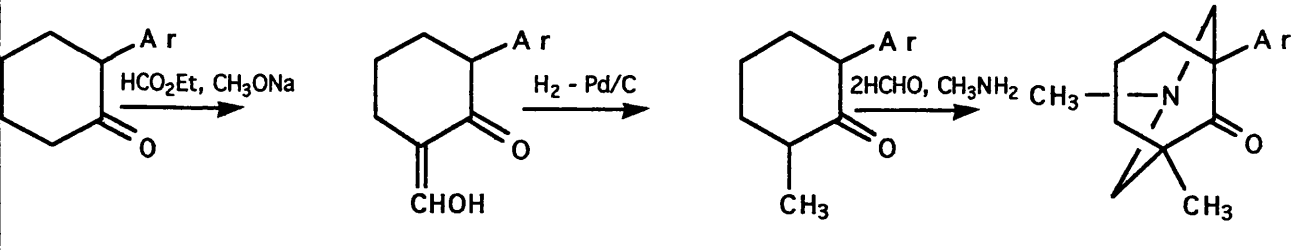
(141) (143) (144) (145)



(146) (147) (148)



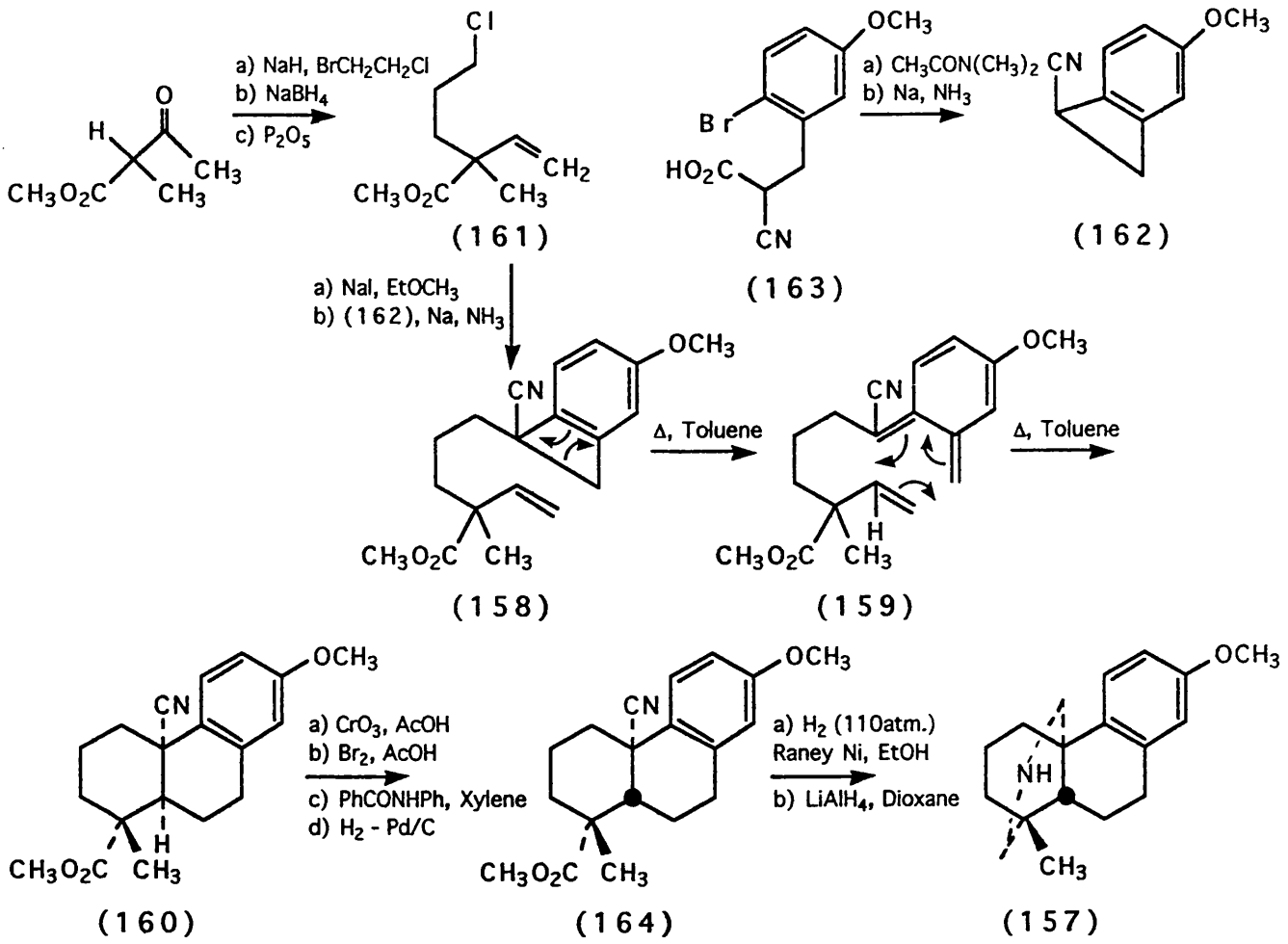
(149) (151) (152) (150)

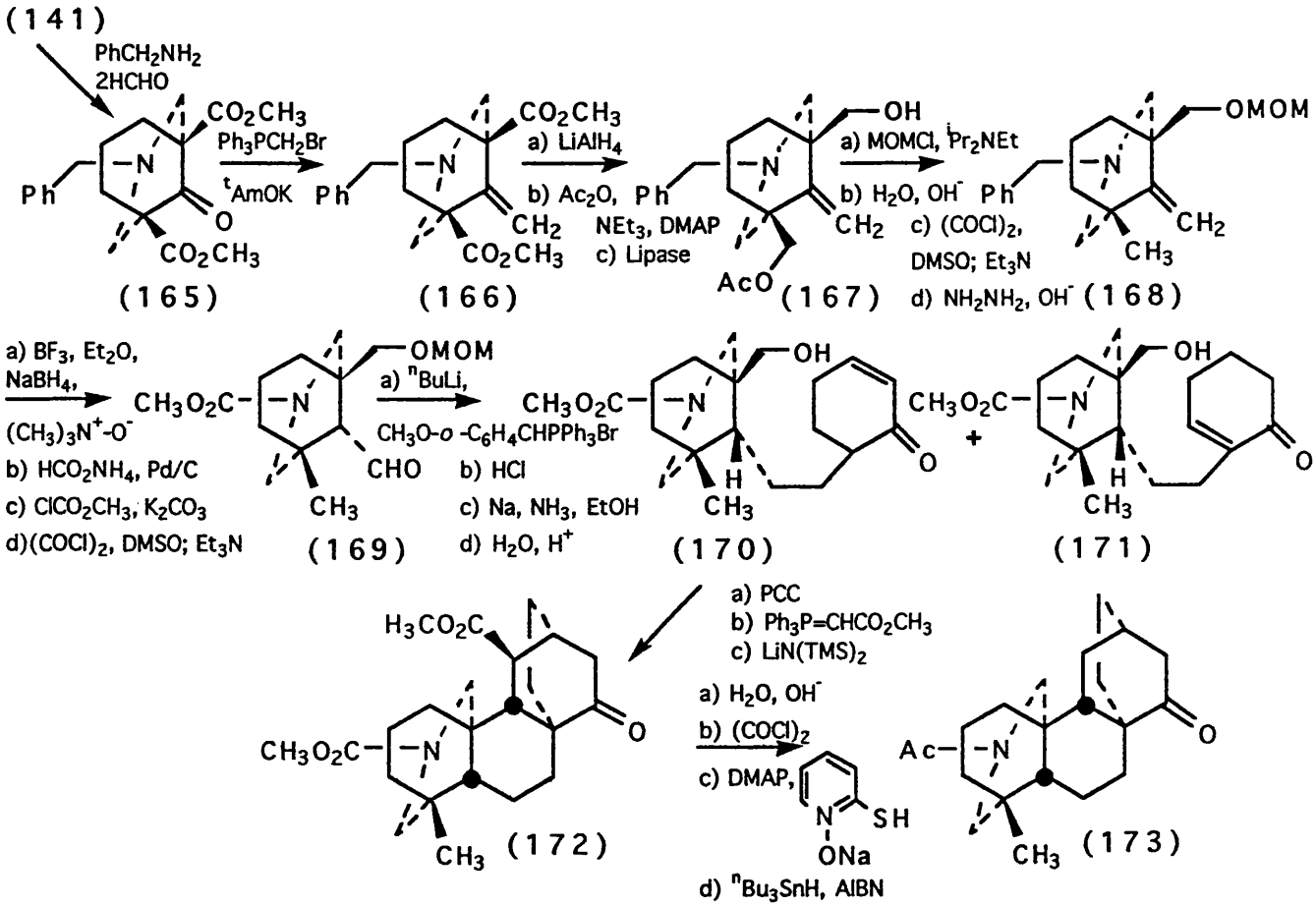


(153) (155) (154) (156)

The synthesis of intermediate (157) for (±)-atisine (67) was tackled by a group of researchers headed by Fukumoto (Pelletier and Page, 1978 and Kametani *et al.*, 1976), with the key step in this synthesis being the thermolytic intramolecular cycloaddition of (158), *via* (159) to produce (160). [4.2.0]Bicycle (158) was synthesized from methyl methylacetoacetate: alkylation with 1-bromo-3-chloropropane, followed by reduction to the corresponding secondary alcohol and subsequent dehydration gave alkene (161). Condensation of the corresponding iodo compound with (162) [from α-cyanophenylpropionic acid (163)] gave benzocyclobutene (158) as required. This *cis*-fused octalin (160) obtained on thermolysis, was then converted into the *trans*-fused system (164) (by oxidation, bromination, dehydrobromination and hydrogenation). Tetracycle (157), containing the AE-[3.3.1]bicycle, was obtained by reduction of (164) with Raney nickel under 110 atmospheres of hydrogen, followed by treatment with $LiAlH_4$ to reduce the amide carbonyl to a methylene.

Over the period 1986-1994, Ihara *et al.* reported the stereocontrolled formal synthesis of (±)-atisine (67) starting from dimethyl cyclohexanone-2,6-dicarboxylate (141) (R = methyl), which was used in a double Mannich condensation to give the symmetrical azabicyclo[3.3.1]nonane (165) (AE ring system) (Ihara *et al.*, 1990a). Reaction of (165) with the appropriate ylid yielded (166) with an *exo* methylene. Reduction of this diester ($LiAlH_4$) afforded the corresponding diol which was then converted into the diacetate. Enantioselective acylation, catalyzed with lipase, a key step in the synthetic route, gave the (+)- and (-)-forms of monoacetate (167) [(+)-form shown]. In order obtain intermediate (168), it was necessary to treat the (+)- and (-)-optically pure forms of (167) slightly differently. In the case of the former, protection of the alcohol group (with methoxymethyl chloride) was needed, prior to basic hydrolysis of the acetyl group, Swern oxidation and treatment with alkaline hydrazine, whereas the alcohol group of other enantiomer was immediately converted into a methyl group via aldehyde (in the same way),

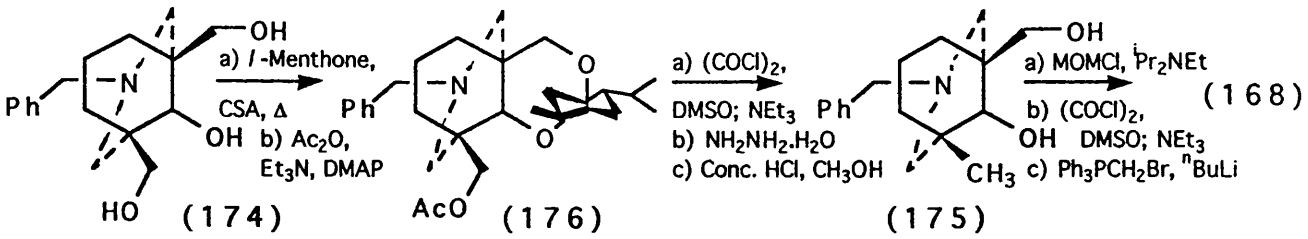

a) NaH, BrCH2CH2Cl
b) NaBH4
c) P2O5
(161)
a) CH3CON(CH3)2
b) Na, NH3
(162)
(163)
a) NaI, EtOCH3
b) (162), Na, NH3
Δ, Toluene
Δ, Toluene
(158)
(159)
a) CrO3, AcOH
b) Br2, AcOH
c) PhCONHPh, Xylene
d) H2 - Pd/C
a) H2 (110atm.)
Raney Ni, EtOH
b) LiAlH4, Dioxane
(160)
(164)
(157)

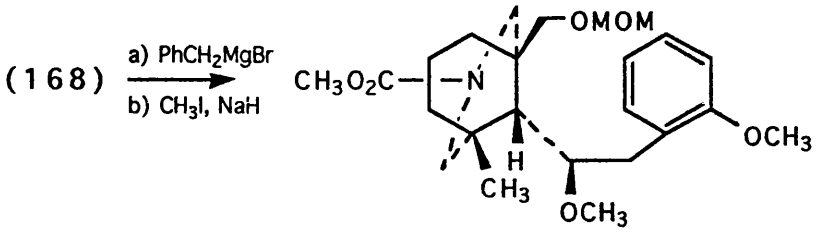

(141)
PhCH2NH2
2HCHO
Ph3PCH2Br
tAmOK
(165)
a) LiAlH4
b) Ac2O, NEt3, DMAP
c) Lipase
(166)
(167)
a) MOMCl, iPr2NEt
b) H2O, OH-
c) (COCl)2, DMSO; Et3N
d) NH2NH2, OH-
(168)
a) BF3, Et2O, NaBH4,
(CH3)3N+-O-
b) HCO2NH4, Pd/C
c) ClCO2CH3, K2CO3
d)(COCl)2, DMSO; Et3N
(169)
a) nBuLi,
CH3O-o-C6H4CHPPh3Br
b) HCl
c) Na, NH3, EtOH
d) H2O, H+
(170)
(171)
a) PCC
b) Ph3P=CHCO2CH3
c) LiN(TMS)2
a) H2O, OH-
b) (COCl)2
c) DMAP,
d) nBu3SnH, AIBN
(172)
(173)

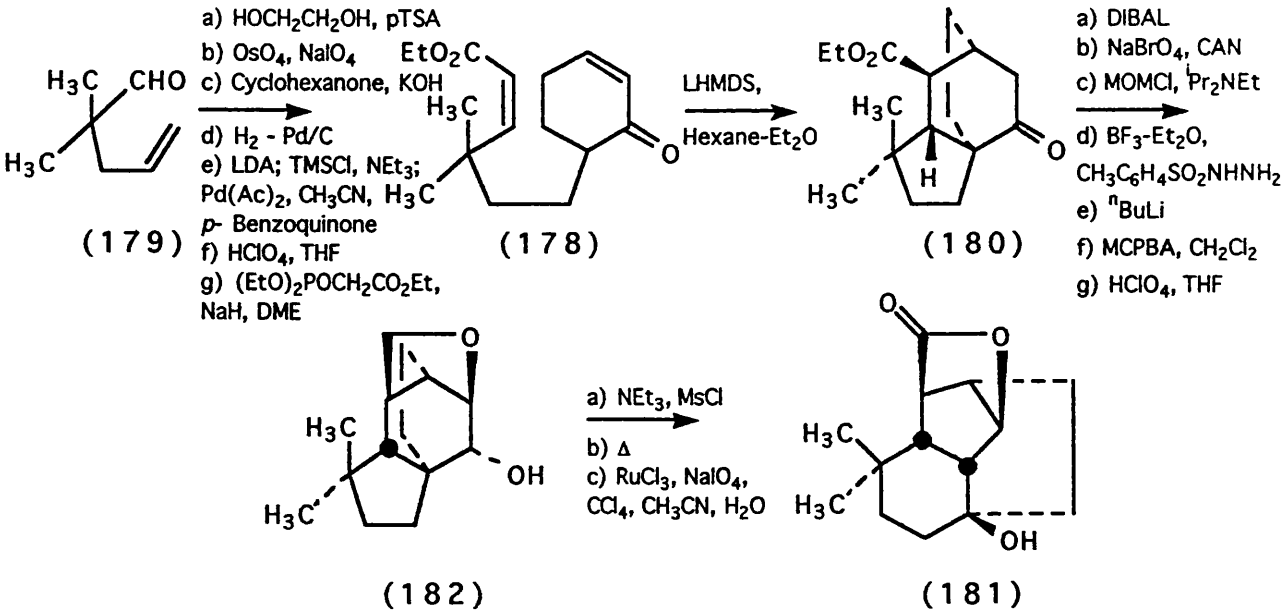
followed by methoxymethyl protection to give the required product (168). Stereoselective hydroboration of the double bond of (168) was then achieved to give primary alcohol. It was then necessary to remove the *N*-benzyl group (hydrogenolysis) and to reprotect the secondary amine as a methyl carbamate. This was efficiently converted, by Swern oxidation, into (169) which is then used in a Wittig reaction [(2-methoxybenzyl)triphenylphosphonium ylid]. This styrene (*trans* isomer) is methoxymethyl deprotected and subjected to Birch reduction was followed by acid hydrolysis to give enones (170) and (171).
An α,β-unsaturated ester group was then introduced, by Swern oxidation and Wittig reaction, in order to perform the intramolecular double Michael reaction to construct the spirofused [2.2.2]octane (CD ring system) (172). Decarboxylation by Barton's free-radical procedure followed by conversion of the carbamate into an acetamide function gave the key bridged pentacyclic intermediate (173) which has been correlated with atisine (67).

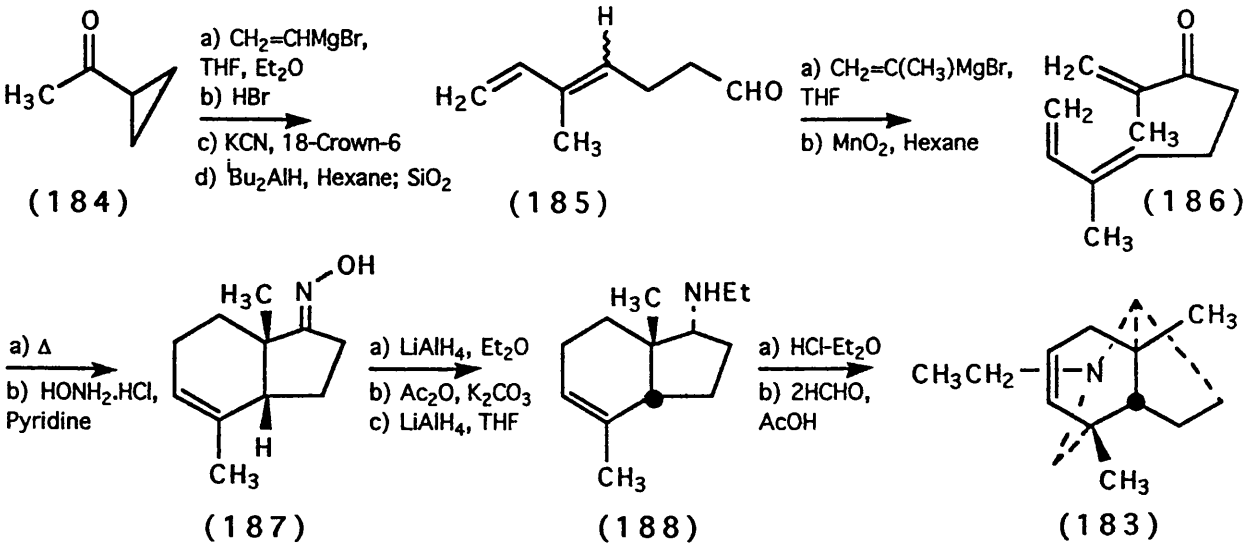
Ihara *et al.* also described another method for the synthesis of alkene (168) from (165) (Ihara *et al.*, 1990b). Reduction of (165) with lithium triborohydride, followed by benzoylation, separation of the stereoisomers by chromatographic methods and debenzoylation, furnished triol (174). Conversion into diol (175) was then achieved by using *l*-menthone protection followed by separation of the corresponding acetates [(176) 1 only shown] prior to oxidation of the alcohol function to an aldehyde group [at C(4)], followed by Wolff-Kishner reduction and removal of the chiral auxiliary to give methyl derivative (175). The required compound (168) was then easily obtainable by methoxymethyl protection to give the corresponding secondary alcohol for Swern oxidation and subsequent Wittig reaction of the resulting ketone.

In 1992, the same research group reported the enantioselective synthesis of 6-oxygenated atisine derivatives. Oxygen functionalities are common in ring B of diterpenoid alkaloids and it was hoped that, in this case, the oxygen containing


a) I-Menthone, CSA, Δ
b) Ac2O, Et3N, DMAP
a) (COCl)2, DMSO; NEt3
b) NH2NH2.H2O
c) Conc. HCl, CH3OH
a) MOMCl, iPr2NEt
b) (COCl)2, DMSO; NEt3
c) Ph3PCH2Br, nBuLi
(174)
(176)
(175)
(168)


(168)
a) PhCH2MgBr
b) CH3I, NaH
(177)


a) HOCH2CH2OH, pTSA
b) OsO4, NaIO4
c) Cyclohexanone, KOH
d) H2 - Pd/C
e) LDA; TMSCl, NEt3; Pd(Ac)2, CH3CN, p- Benzoquinone
f) HClO4, THF
g) (EtO)2POCH2CO2Et, NaH, DME
LHMDS, Hexane-Et2O
a) DIBAL
b) NaBrO4, CAN
c) MOMCl, iPr2NEt
d) BF3-Et2O, CH3C6H4SO2NHNH2
e) nBuLi
f) MCPBA, CH2Cl2
g) HClO4, THF
a) NEt3, MsCl
b) Δ
c) RuCl3, NaIO4, CCl4, CH3CN, H2O
(179)
(178)
(180)
(182)
(181)


a) CH2=CHMgBr, THF, Et2O
b) HBr
c) KCN, 18-Crown-6
d) iBu2AlH, Hexane; SiO2
a) CH2=C(CH3)MgBr, THF
b) MnO2, Hexane
a) Δ
b) HONH2.HCl, Pyridine
a) LiAlH4, Et2O
b) Ac2O, K2CO3
c) LiAlH4, THF
a) HCl-Et2O
b) 2HCHO, AcOH
(184)
(185)
(186)
(187)
(188)
(183)

functional group would assist in C(7)-C(20) bond formation. The synthesis used the same intramolecular double Michael approach as described above with the oxygen functionality being introduced by means of a Grignard reaction between benzylmagnesium bromide and aldehyde (169) followed by methylation and separation of the diastereoisomers formed [(177) 1 only shown].

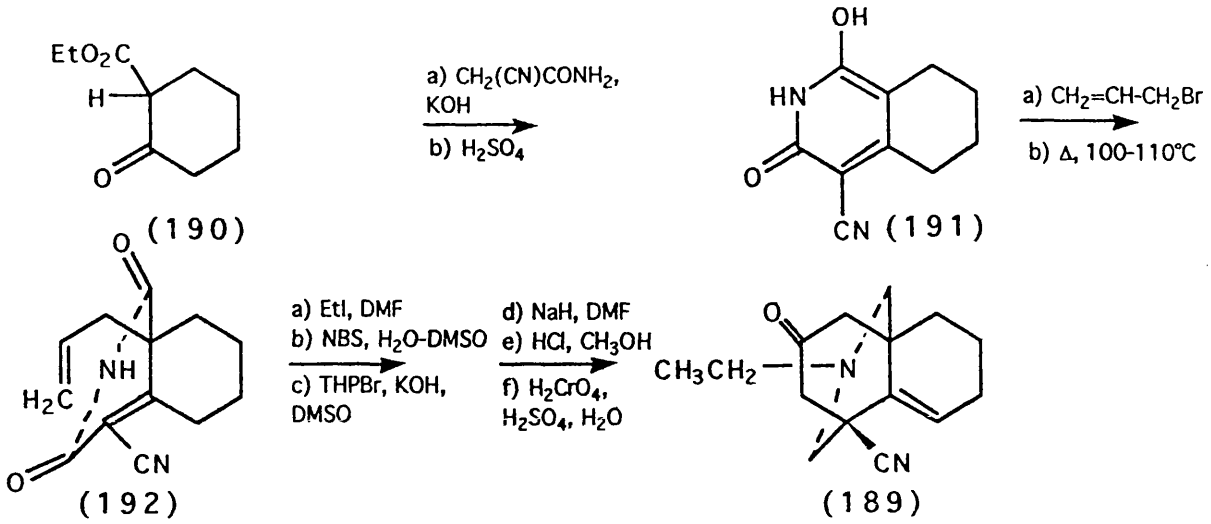
Fukumoto and co-workers considered a model study towards the total synthesis of *Aconitum* alkaloids and in particular the BCD ring system of the lycoctonine skeleton. Intramolecular double Michael reaction of α,β-unsaturated enone ester (178) [from 2,2-dimethylpent-4-enal (179)] produced tricyclic ketone (180), which was converted into lactone (181) *via* tetracyclic alcohol (182), using a Wagner-Meerwein rearrangement as the key step in constructing this BCD tricycle analogue (Ihara *et al.*, 1986 and 1988).

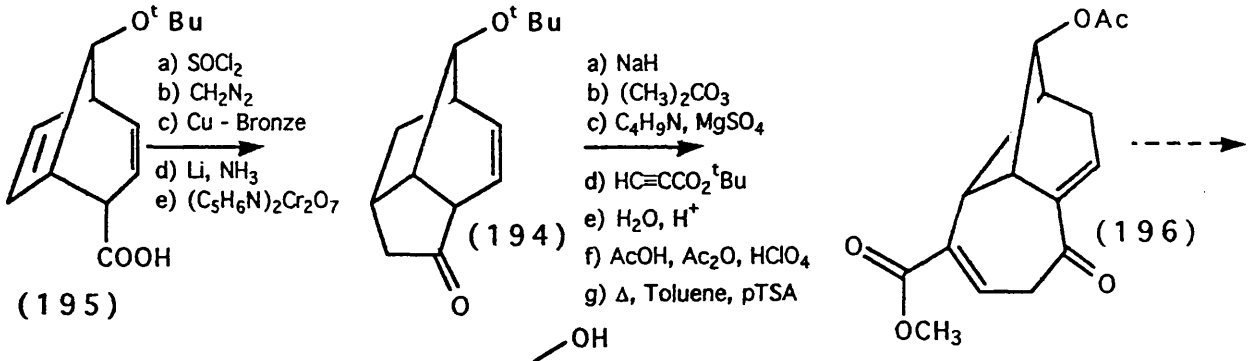
The synthesis of enamine (183) (AEF ring system of diterpenoid alkaloids) was successfully carried out by Shishido *et al.* (1986 and 1989) starting from cyclopropyl methyl ketone (184). Aldehyde (185) was obtained in four steps (Grignard reaction, bromination, conversion into the corresponding cyanide compound and reduction) and was then converted into a mixture of *cis* and *trans* isomers of (186) by another Grignard reaction and oxidation of the resulting secondary alcohol to the required ketone. Thermolysis of trienone (186), followed by treatment of the cycloadducts with hydroxylamine furnished a separable mixture of *cis* and *trans* fused oximes [(187) 1 only shown]. The corresponding acetamide was obtained (reduction then acetylation), which was then reduced to give secondary amine (188). Finally, a Mannich-type reaction, performed on the hydrochloride of (188) gave the expected tricycle (183) as its hydrochloride in a completely regioselective manner.

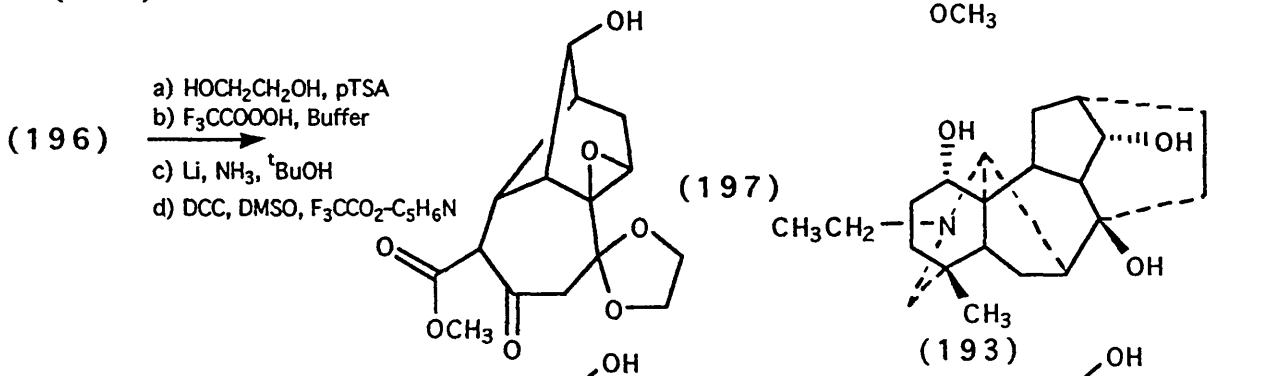
A novel approach to the ABE ring system (189) of C_{20}-diterpenoid alkaloids was reported in 1975 by van der Baan and co-workers (within Pelletier and Page, 1978). Cyclic ketoester (190) was treated with cyanacetamide to give (191). Then *C*-alkylation with allyl bromide followed by a Cope-type rearrangement afforded unsaturated imide (192). *N*-Ethylation, cyclization (using protection) and finally, Jones oxidation gave the required (189).

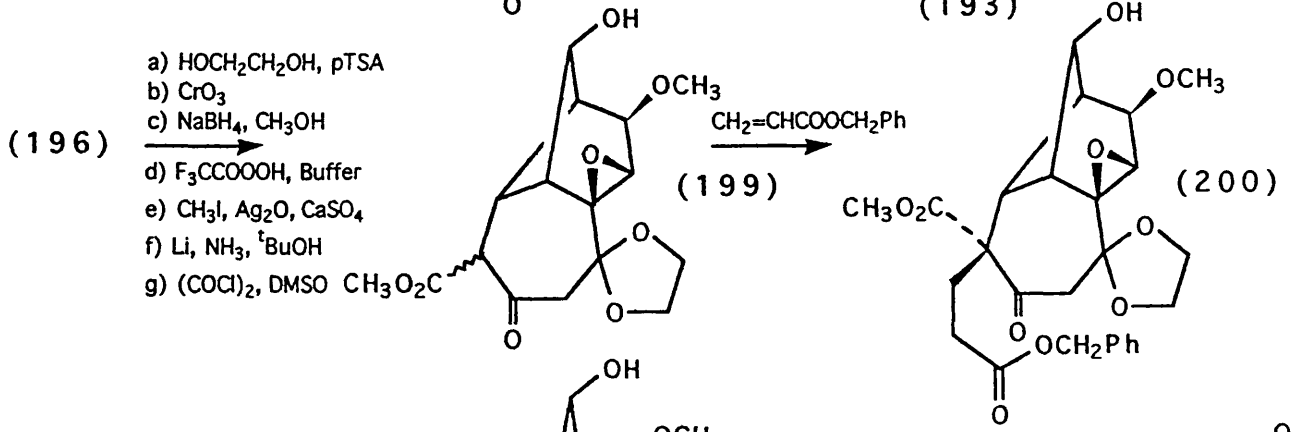
In 1986, the same research group reported (van Beek *et al.*, 1986) the construction of the BCD ring system of C_{19}-diterpenoid alkaloids such as cardiopetaline (193). The 5-membered ring ketone (194) [from 7-*tertiary*-butoxy-norbornadiene *via* (195)] was used to prepare the 7-membered ring ketone (196). Reaction of (194) with dimethylcarbonate and NaH gave the corresponding β-keto ester which was then converted into the enamino ester. A ring expansion reaction using *tertiary*-butyl propiolate followed by a further three steps (acid hydrolysis, acetylation and decarboxylation) gave (196). Cyclic β-keto ester (197) was then efficiently prepared using ethylenedioxy protection of the keto group at C(8), epoxidation of both double bonds, conversion of the epoxy ester into β-hydroxy ester with lithium and *tertiary*-butanol in liquid ammonia and oxidation using either Pfitzner-Moffat or Swern methods.

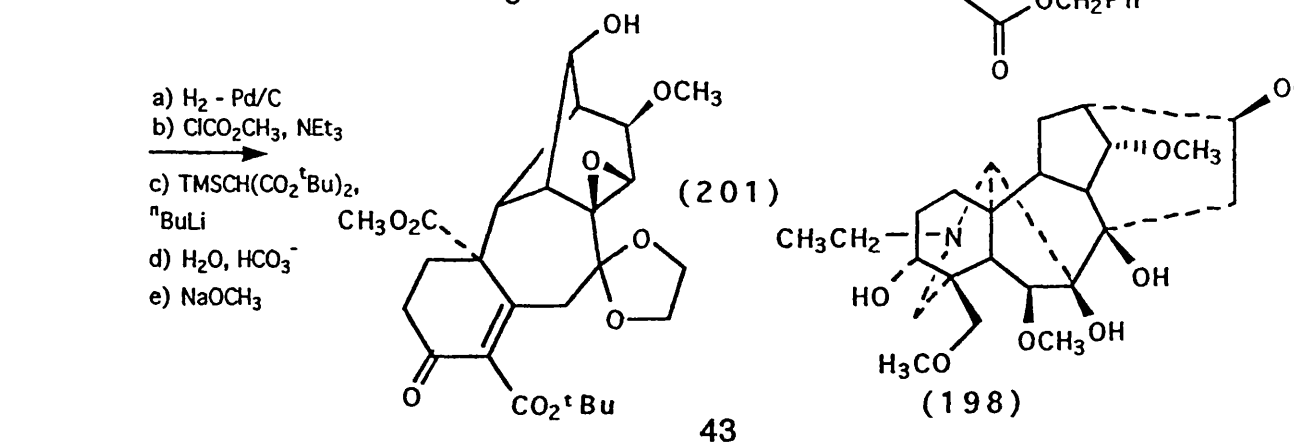
In 1992, van der Baan and co-workers reported (van der Baan *et al.*, 1992) their successful incorporation of a methoxy group [at C(16), as found in C_{19}-diterpenoid alkaloids such as acomonine (198)] into the BCD tricyclic intermediate (199). This was achieved by oxidation of the ethylenedioxy protected compound of (196) with chromium trioxide complexes and then reduction of the resulting ketone to give a mixture of alcohols. The same approach as described previously was used to introduce the appropriate substituents into the CD skeleton (epoxidation preceded methylation of the alcohols and was followed by the lithium in liquid ammonia reaction) and the


EtO2C
H
O
(190)
a) CH2(CN)CONH2, KOH
b) H2SO4
OH
HN
O
CN (191)
a) CH2=CH-CH2Br
b) Δ, 100-110°C
O
NH
H2C
CN
O
(192)
a) EtI, DMF
b) NBS, H2O-DMSO
c) THPBr, KOH, DMSO
d) NaH, DMF
e) HCl, CH3OH
f) H2CrO4, H2SO4, H2O
CH3CH2
N
CN
(189)


O^t Bu
a) SOCl2
b) CH2N2
c) Cu - Bronze
d) Li, NH3
e) (C5H6N)2Cr2O7
COOH
(195)
O^t Bu
(194)
a) NaH
b) (CH3)2CO3
c) C4H9N, MgSO4
d) HC≡CCO2tBu
e) H2O, H+
f) AcOH, Ac2O, HClO4
g) Δ, Toluene, pTSA
OAc
(196)
OCH3


(196)
a) HOCH2CH2OH, pTSA
b) F3CCOOOH, Buffer
c) Li, NH3, tBuOH
d) DCC, DMSO, F3CCO2-C5H6N
OH
O
OCH3
(197)
OH
OH
CH3CH2
N
OH
CH3
(193)


(196)
a) HOCH2CH2OH, pTSA
b) CrO3
c) NaBH4, CH3OH
d) F3CCOOOH, Buffer
e) CH3I, Ag2O, CaSO4
f) Li, NH3, tBuOH
g) (COCl)2, DMSO
OH
OCH3
CH3O2C
(199)
CH2=CHCOOCH2Ph
OCH2Ph
(200)

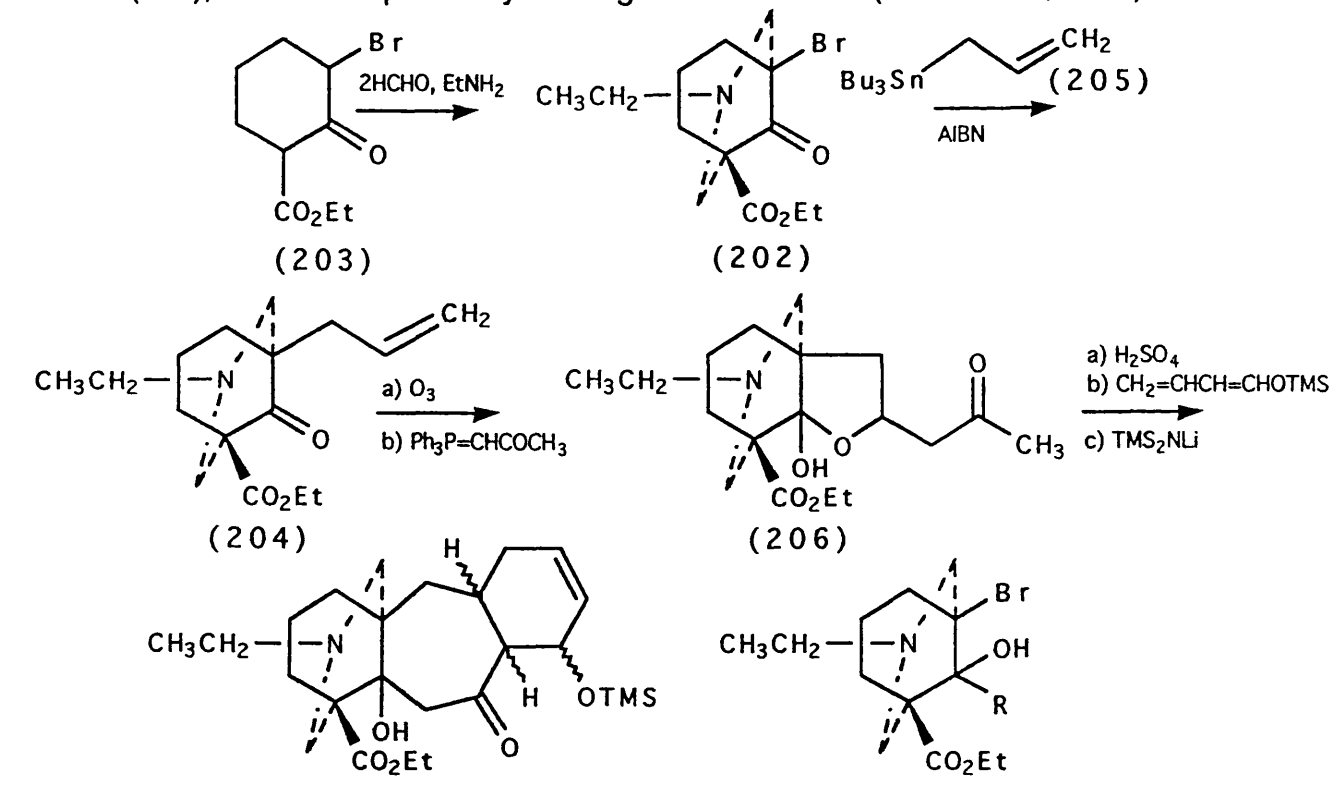

a) H2 - Pd/C
b) ClCO2CH3, NEt3
c) TMSCH(CO2tBu)2, nBuLi
d) H2O, HCO3-
e) NaOCH3
OH
OCH3
CH3O2C
CO2tBu
(201)
CH3CH2
N
HO
OCH3
H3CO
OH
(198)

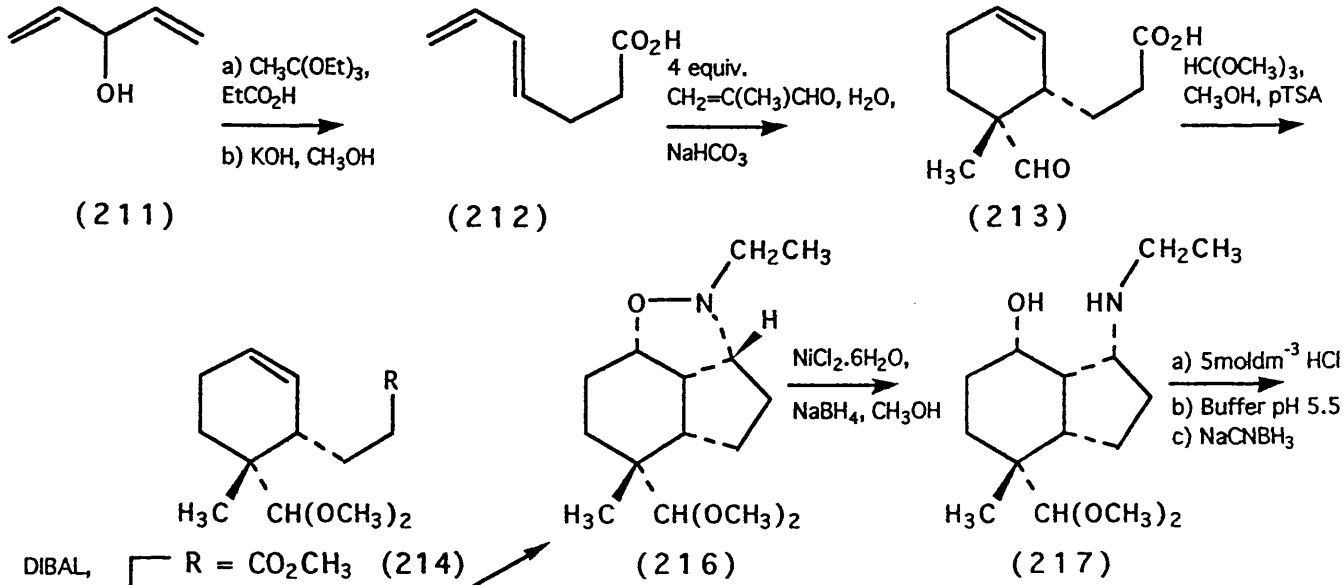
β-keto ester (199) was reacted in a Michael addition with benzyl acrylate to give (200). The protecting group was removed by hydrogenolysis and the resulting carboxylic acid converted, *via* a mixed anhydride into the corresponding *tertiary*-butyl β-keto ester. Cyclization of ring A and removal of the acetyl protection was effected by treatment with sodium methoxide, giving the required tetracycle (201).

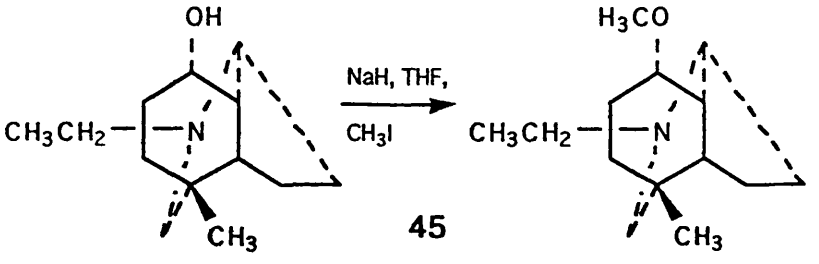
The approach of Kraus and Shi (1990) towards diterpenoid alkaloids involved the preparation of the AE ring system (202) from keto diester (141) (R = ethyl) *via* bromide (203), using a double Mannich condensation. This research group investigated (Kraus *et al.*, 1993) the bridgehead radical reactivity of (203) and were able to isolate (204) using allylic tin reagent (205). Ozonolysis of (204), followed by Wittig reaction unexpectedly afforded hemiketal (206). This was quantitatively converted into the corresponding enone before Diels-Alder reaction with 1-trimethylsiloxy-1,3-butadiene and intramolecular aldol condensation (potassium hexamethyldisilazane as base) furnished AEBD-tetracycle (207). Kraus and Shi (1991) also reported the conversion of (203) into alcohol (208) and (209), using the appropriate Grignard reagents. It is noteworthy that alkyl lithium reagents, enolate anions and phosphonate anions all generated rearranged alcohols with azabicyclo[3.3.0]octane skeletons.

Tricyclic amine (210), representing the AEF ring system of MLA has been synthesized from penta-1,4-dien-3-ol (211) in nine steps by Baillie *et al.* (1994). Heating with triethyl orthoacetate, followed by alkaline hydrolysis of the resulting ester gave diene (212). *Endo* cyclohexene acid (213) was obtained on Diels-Alder reaction of the sodium salt of (212) with methacrolein. Simultaneous acetalization and esterification gave ester (214), for reduction to aldehyde (215). Isoxazolidine (216) was afforded on reaction with ethyl-hydroxylamine. Cleavage of the N-O bond gave amine (217), followed by closure of the piperidine ring, afforded tricycle (218). *O*-Methylation provided

the desired product (210). The *N*-methylated series, for the scheme (216) - (210), was also reported by Whiting and co-workers (Baillie *et al.*, 1994).







CHAPTER 2

NATURAL PRODUCTS ISOLATION, CHARACTERIZATION AND SEMISYNTHESIS

2.1 AIMS

The aims of these phytochemical investigations were:

i) to obtain and characterize pure MLA (1) in sufficient quantity for biological studies. This involved the optimization of extraction and isolation procedures using seeds of *Delphinium* hybrid cultivars as a source of alkaloid.

ii) to establish unambiguously the configuration of the methyl succinimide moiety, the only undefined chiral centre in MLA, by synthesis of *S*-(-)-methylsuccinic acid and its dimenthyl ester and comparison of the optical rotation and ^{13}C NMR spectra of those with that obtained by hydrolysis of natural MLA.

iii) to isolate and characterize other natural alkaloids co-occurring with MLA.

iv) to obtain pure lycoctonine in sufficient quantity for semi-synthetic acylation.

v) to synthesize semi-synthetic alkaloids from lycoctonine.

2.2 RESULTS AND DISCUSSION

2.2.1 Optimization of the Extraction of Garden Hybrid *Delphinium* Seeds

As part of our SAR studies, we have undertaken the isolation and characterization of norditerpenoid alkaloids from Garden Hybrid *Delphinium*, the common giant perennial *Delphinium* of horticulture which is closely similar to the American cultivar, *Delphinium* Pacific Giant (*D. elatum*). In our preliminary experiments, we particularly aimed to determine whether the seeds of Garden Hybrid *Delphinium* contain adequate amounts of MLA (1). It was important to optimize the extraction of Garden Hybrid *Delphinium* seeds, to produce consistently a maximum yield of crude alkaloidal material, whilst minimizing the amount of time and solvents required. The seeds (as opposed to the roots, leaves, flowers or other aerial portions) of *Delphinium* are considered to be the most alkaloid rich; seed extraction can yield as much as 2% alkaloidal material (Ross *et al.*, 1988). However, values of 0.2-0.6% of the total dry weight of the plant are more usual (Majak *et al.*, 1987 and Pelletier *et al.*, 1981b). A number of seed extractions were carried out on 12g or 20g scales for the purpose examining a traditional literature procedure (Pelletier *et al.*, 1990), exploring the use of a defatting process, investigating the use of a Soxhlet extraction process, considering the efficiency of a number of extraction solvents, and studying the effect of the density of crushed seeds in a Soxhlet extraction.

The traditional extraction process that we followed (Pelletier *et al.*, 1990), involved treating the crushed seeds with an ethanol/water/hexane mixture by agitation, using a mechanical shaker, over a two to three day period. This was followed by taking the extracts through an acid/base cycle which involved a number of involved filtration, separation and partitioning steps as well as evaporations under reduced pressure which were inclined to 'bump' and which

would present many difficulties in larger scale experiments. Therefore, despite the fact that this method yielded crude alkaloidal material amounting to 1.25% of the total weight of seeds taken and used only modest volumes of solvents, the procedure was considered to be laborious and problematical. On scaling-up a procedure such as this, problems might be anticipated with effective stirring and control of the temperature.

The introduction of a defatting process prior to extraction aimed to both optimize the extraction and avoid the practical problems of separating thick emulsions, as experienced in the traditional extraction experiment. The fats and/or oils (triglycerides, involving long-chain saturated or unsaturated acids) which often occur in seeds, are usually insoluble in alcohol and water and soluble in ether and hexane. In our studies, we found hexane to be useful for the removal of these lipids. The clarity of the extracts was used as an indication of effective defatting.

In the traditional extraction process, extraction was performed by repeated maceration with agitation and percolation. The introduction of the use of a Soxhlet continuous extractor aimed to both optimize the extraction and avoid the practical problems of removing large volumes of aqueous ethanol encountered in the traditional extraction experiment. The choice of solvent for the purpose of extraction of the crude bases from our powdered plant material is clearly important. A variety of extraction solvents are widely accepted, including diethyl ether and chloroform, but for many plant constituents, alcoholic solvents are generally used (Kulanthaivel *et al.*, 1986).

In a preliminary Soxhlet experiment, methanol followed by chloroform were used as extraction solvents. However, a disappointing 0.85% overall yield was obtained, possibly due to too rapid cycling of the extraction process, and therefore, in a subsequent extraction, ethanol was substituted for methanol. It was hoped that this more viscous and higher boiling solvent would aid slower and more efficient Soxhlet extraction. Again, a chloroform extraction was necessary after the alcoholic one, but this time the overall yield was 1.23%,

which is comparable to the traditional extraction process. This two step Soxhlet method revealed no real advantages over the traditional extraction process, involving at least as much labour and time and considerably larger volumes of solvents, but it did at least indicate that the alkaloids were sufficiently heat stable for Soxhlet extraction.

Accordingly, the process was simplified by using chloroform as the sole solvent, which also allowed the use of a single acid/base cycle for subsequent purification of the alkaloids from non-alkaloidal constituents (See Section 2.3.2.1). In the hope that the penetration of the extraction solvent might be increased and thus the yield of alkaloids maintained at approximately 1.25% or better, in this extraction the density of the seed packed into the Soxhlet thimble was decreased (12g as opposed to 20g of ground seeds were placed in the same size thimble). Another experiment, based on the same principle, involved mixing the crushed seeds with small glass balls as packing, but no improvement in the yield over the less densely packed seeds case was observed and the glass balls were, therefore, deemed unnecessary. The yields obtained by the chloroform-only extractions were 1.22%, which are consistent with the previous experiments, implying that the maximum amount of alkaloid is, in each case, being obtained. This single step, reduced density Soxhlet extraction was found to be substantially less labour intensive, without using excessive solvent and without sacrificing yields.

A number of TLC systems for analysizing the crude alkaloidal material were investigated. However, the TLC eluant found to give the best separation of the alkaloids in the crude material was 5:4:1 cyclohexane-chloroform-diethylamine as used by Jennings *et al.* in 1986, with detection by Dragendorff Munier spray (See Section 2.3 for experimental details). Our TLC analysis indicated the presence of three major and at least three minor alkaloid components with comparable chromatograms being obtained for each of the extraction methods.

^{1}H NMR spectroscopy of the crude material exhibited promising peaks in the aromatic region indicating the presence of a 1,2-disubstituted benzene ring as seen in MLA (1) and related alkaloids [δ8.05 (d), δ7.70 (t), δ7.55 (t), and δ7.30 (d)ppm]. Four peaks between δ5.05 and δ5.25ppm in the spectrum obtained indicate the presence of alkaloids containing methylenedioxy functions between C-7 and C-8 [δ5.22 (s), δ5.13 (s), δ5.10 (s), and δ5.05 (s)ppm]. About fifty norditerpenoids possessing this moiety have been isolated from *Delphinium* (Pelletier *et al.*, 1981b). Identical ^{1}H NMR spectra were obtained for each of the extraction methods.

The extraction method described in Section 2.3.2.1, using a reduced density of seeds in the Soxhlet thimble and chloroform as solvent was scaled up to a 300g extraction, allowing approximately 4g (1.27%) crude alkaloidal material to be obtained in three to four days.

2.2.2 Purification of Crude Alkaloidal Material

Preparative thin layer chromatography (pTLC), which has been popular with many research groups for a long time for the purification of mixtures of alkaloids and the less time-consuming modern chromatographic techniques of centrifugal chromatography (Desai *et al.*, 1985 and 1986) and vacuum liquid chromatographic (VLC) (Pelletier *et al.*, 1986b) were compared in order to develop a suitable purification method for our compounds.

Only partial separation of the alkaloids contained in the off-white foam mixture was achieved by pTLC, yielding three alkaloids amounting to less than 50% of the crude alkaloid applied (See Section 2.3.2.2). The least polar fraction (Fraction 1) was found to contain mainly one alkaloid and the peaks at δ5.05 and δ5.13ppm in the ^{1}H NMR spectrum indicated that this is one of the alkaloids picked out from the crude mixture as possessing a methylenedioxy function

between C-7 and C-8 (See later in Section 2.2.3). The middle band (Fraction 2) again appeared to contain one major component. The ^{1}H NMR spectrum (See later in Section 2.2.5) of this alkaloid showed clearly a dd, dt, dt, dd pattern in the aromatic region of the spectrum, as seen for an authentic sample of MLA. citrate. A polar fraction (Fraction 3) was found to contain more than one component, but the major alkaloid present appears again to possess a methylenedioxy function on examination by ^{1}H NMR. Spectroscopic techniques were used to elucidate the structures of both of the pure isolated alkaloids (Fraction 1 and Fraction 2) and full spectral data are discussed later in Section 2.2.3 and Section 2.2.5 but insufficient pure material for complete analysis of the alkaloids in Fraction 3 was generated.

All three of the fractions obtained by pTLC showed peaks in the ^{1}H NMR spectra in the aromatic region (dd, dd, t, t) and the aliphatic region which were assigned to dibutyl phthalate, a common plasticizer.

Centrifugal chromatography has been used by other research groups as a technique for purification of mixtures of norditerpenoid alkaloids (Desai *et al.*, 1985 and 1986). It has several advantages over pTLC - as well as being at least as efficient, it is less tedious, time-consuming and expensive and it is better suited to large scale purifications (Marston and Hostettmann, 1991). Fractionation of the crude alkaloid extracts obtained from the seeds was, therefore, attempted using a chromatotron (See Section 2.3.2.3). However, using the same solvent system as TLC and pTLC, approximately half of the material applied was recovered as a mixture, possibly because the eluting solvent was too polar. However, both of the less polar components obtained by pTLC were again obtained pure (^{1}H NMR and TLC), in small quantities, by this technique.

VLC is another modern chromatographic technique that has recently been used for the separation of complex alkaloid mixtures (Pelletier *et al.*, 1986b). This

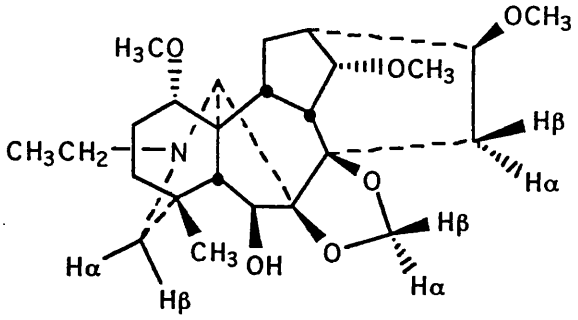
method is superior to pTLC in that it enables the separation of gram quantities in a single run. In our hands, it was found to be effective, rapid and economical for purification of both large and small quantities of crude material. Using VLC 100mg of the methylenedioxy containing alkaloid (the least polar component) and 380mg of the MLA-like component (the second band by TLC) were isolated from approximately 1g of crude alkaloidal material, using an alumina bed and eluting with a gradient of hexane, diethyl ether and methanol (See Section 2.3.2.4). This experiment indicated that the second component isolated may account for as much as approximately 35-50% of the alkaloids present.

Accordingly, having assessed a variety of purification methods, the VLC procedure (or a combination of VLC and crystallization) was utilized for all subsequent alkaloid purifications.

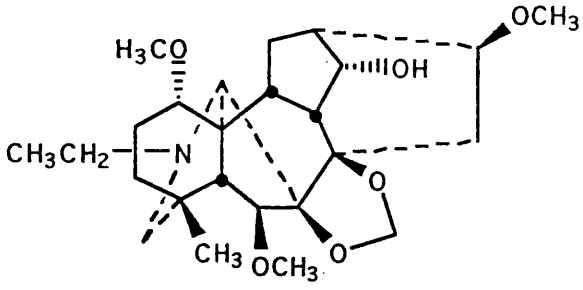
2.2.3 NMR Assignment of Delpheline

In order to achieve unambiguous characterization of the least polar alkaloid (Fraction 1), a crystalline sample was used to obtain a comprehensive set of spectroscopic data (See Section 2.3.2.5). Figures (1) and (2) show the ^{1}H NMR spectrum (400MHz), Figure (3) is the DEPT spectra (100.4MHz) and the HETCOR spectrum is Figure (4) (the COLOC spectrum was also obtained but isn't shown here). Figures (5) and (6) show the COSY and long-range COSY spectra and Figure (7) the phase-sensitive NOESY spectrum. For delpheline [which has been reported from *Delphinium* Pacific Giant, *Delphinium elatum, Delphinium barbeyi, Delphinium occidentale* and *Delphinium ternatum* (Pelletier and Joshi, 1991), the first two of which are related to the hybrid of *Delphinium* extracted in these experiments] the DEPT spectra [Figure (3)] indicated the presence of four quaternary, nine methine, seven methylene, and five methyl carbons and the ^{1}H NMR spectrum [Figures (1) and (2)] revealed three methoxyl signals and peaks at δ5.05 and δ5.13ppm, which are consistent

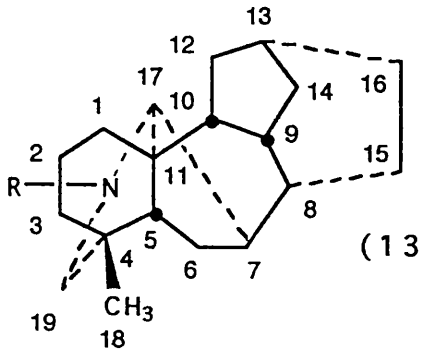
with a methylenedioxy function between C-7 and C-8, and the mass spectrum are broadly in agreement with the data reported for the alkaloid delpheline (219). However, there are some inconsistencies in the ^1H NMR assignments reported in the literature for delpheline (219) (Bando *et al.*, 1989 and Joshi *et al.*, 1991), so it was necessary to carry out a more detailed examination of the NMR data, in order to be confident of the substitution sites.



(219) Delpheline

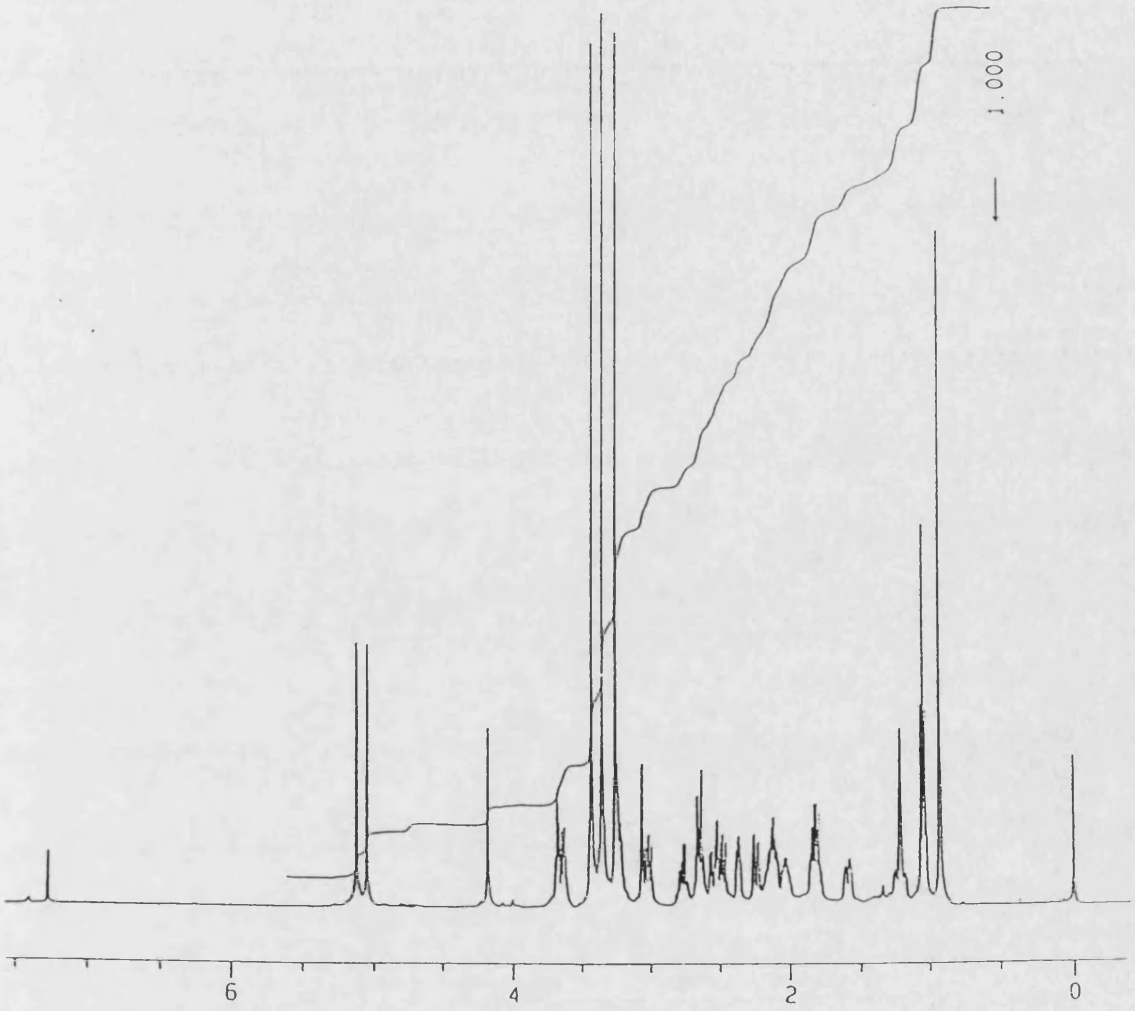


(220) Isodelpheline



(13)

Figure (1) ^1H NMR (400MHz) Spectrum of Delpheline (219) in $CDCl_3$



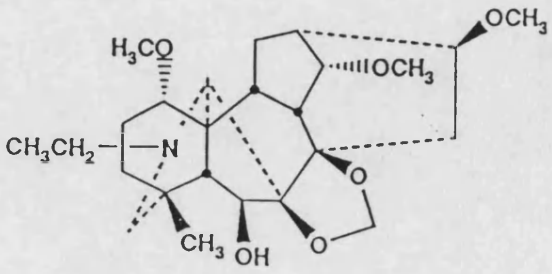
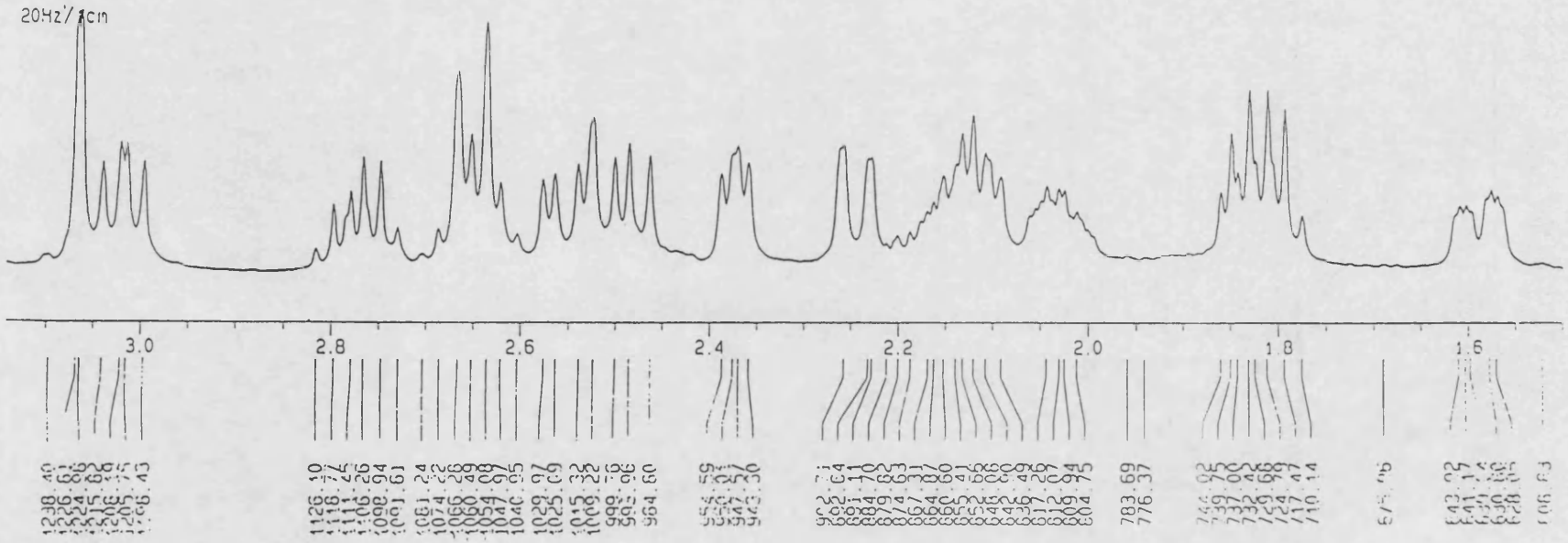


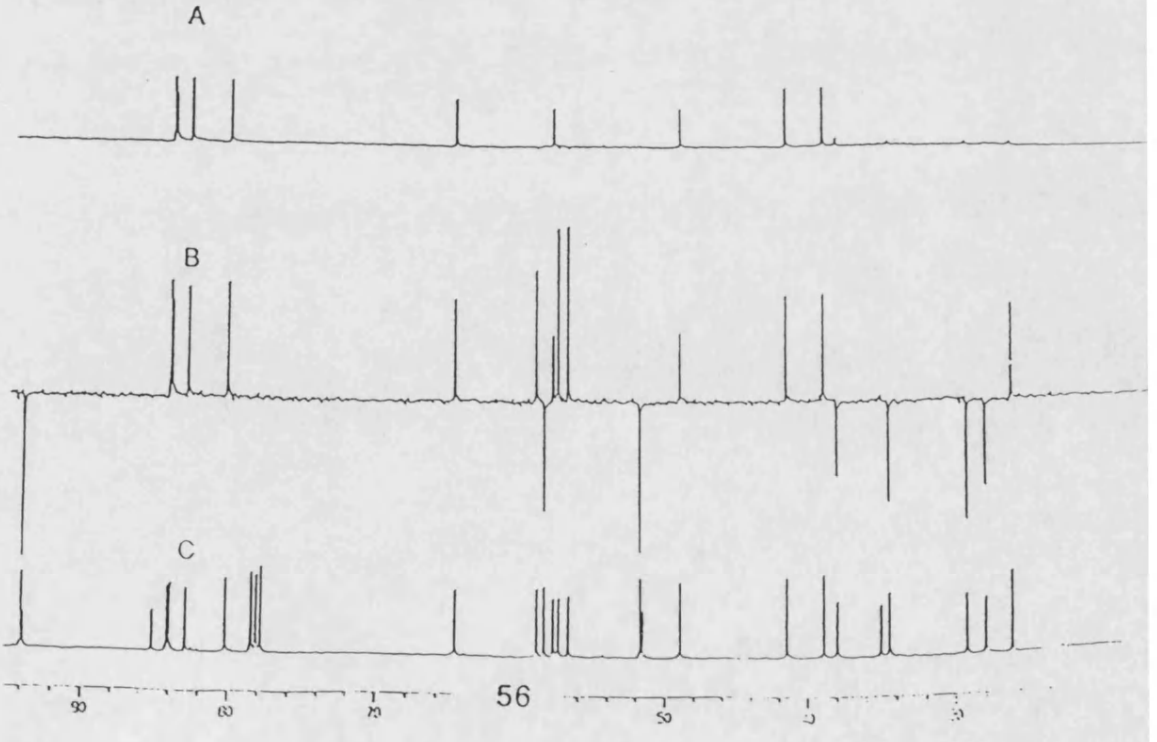
Figure (2) Expansion of ^1H NMR (400MHz) Spectrum of Delpheline (219) in $CDCl_3$



Hanuman and Katz (1994) and Pelletier *et al.* (1984) have collated recent ^{13}C and ^{1}H NMR data, and thus by averaging over a large number of compounds having similar structural features, the general ranges in chemical shifts for the various skeletal and functional groups are available, in tabulated form, and were useful in establishing the structures of these alkaloids (See Section 2.2.8).

Our spectral data for delpheline (219), which are summarized in **Table 1**, allowed us to confidently assign C(1)O*C*H_3 [by observing three bond interactions between C-1 and C(1)O*C*H_3 and C(1)O*C*H_3 and H-1 in a COLOC experiment]. This was possible because the assignment of H-β-1 is undisputed due to the coupling observed to other ring A protons in the COSY spectrum (400MHz), Figure (5)]. Likewise, C(16)-β-O*C*H_3 was assigned, based on the unambiguous assignment of H-α-16 (δ3.25-3.19ppm) by COSY cross-spots with other ring D protons (H-α-15 and H-β-15) [Figure (5)]. The COLOC experiment showed that H-16 was coupled to 56.3ppm [C(16)O*C*H_3] and the carbon at 81.8ppm (C-16) interacted with δ3.35ppm [C(16)OCH_3].

Figure (3) ^{13}C NMR (100.4MHz) Spectra of Delpheline (219) in $CDCl_3$: DEPT 90° Subspectrum showing the Methine Carbons **(A)**, DEPT 135° Subspectrum showing Methine and Methyl Carbons **Up** and Methylene Carbons **Down** (B), and Full Spectrum **(C)**



A crosspeak between H-β-14 and C(16)-β-OCH_3 is observed in the NOESY spectrum [Figure (7)]. Thus, ^{1}H NMR and ^{13}C NMR signals [Figure (3)] for each of the three *O*-methyl ethers at positions 14, 16, and 1 in delpheline (219) can be unambiguously assigned at: δ3.43, δ3.35, and δ3.26ppm, 57.8, 56.3, and 55.5ppm respectively. Our spectral data for the C-1 and C-16 methoxy positions are in agreement with the assignments given in the text by Joshi *et al.* (1991) [^{13}C NMR and HETCOR spectra, Figures (3) and (4), respectively]. An unfortunate transposition in their table of results appears to have arisen (Joshi *et al.*, 1991). We can also agree with the important C-1 α-substituent reassignment made by Pelletier and colleagues (Pelletier *et al.*, 1981a) on observing an interaction between H-β-1 and H-β-10 in the NOESY spectrum [Figure (7)] as well as between C(1)-α-OCH_3 and H-α-12.

The C(6)-β-OH signal was assigned to δ3.34ppm based on the NOESY correlation [Figure (7)] with the downfield portion of the signals at approximately δ2.7ppm (H-β-9). The NOESY experiment [Figure (7)] also showed that the methylenedioxy proton at δ5.13ppm interacted with C(6)OH whilst the upfield proton (δ5.05ppm) interacted with both protons at position 15. We assign the former methylenedioxy proton as β, and the latter as α, in contrast to the recent conclusions of Joshi *et al.* (1991).

Detailed comparison of ^{13}C NMR spectrum [Figure (3)] with those published for isodelpheline (220), the C-14-hydroxy, C-6-methoxy regioisomer (Pelletier *et al.*, 1986a and Pelletier and Joshi, 1991) leads us to believe that the isolated base is delpheline (219) and not isodelpheline (220). The data for isodelpheline (220) shows 74.1 (C-14), 83.5 (C-1), and 89.2ppm (C-6) [plus 93.7ppm (OCH_2O)] (Pelletier *et al.*, 1983 and 1986a), whereas for delpheline (219) we observe 83.0 (C-14), 82.7 (C-1), and 79.2ppm (C-6) (indicating that a downfield shift of approximately 10ppm is observed for a methoxy substituted carbon compared to a hydroxy substituted carbon).

Figure (4) HETCOR Spectrum of Delpheline (219) in $CDCl_3$

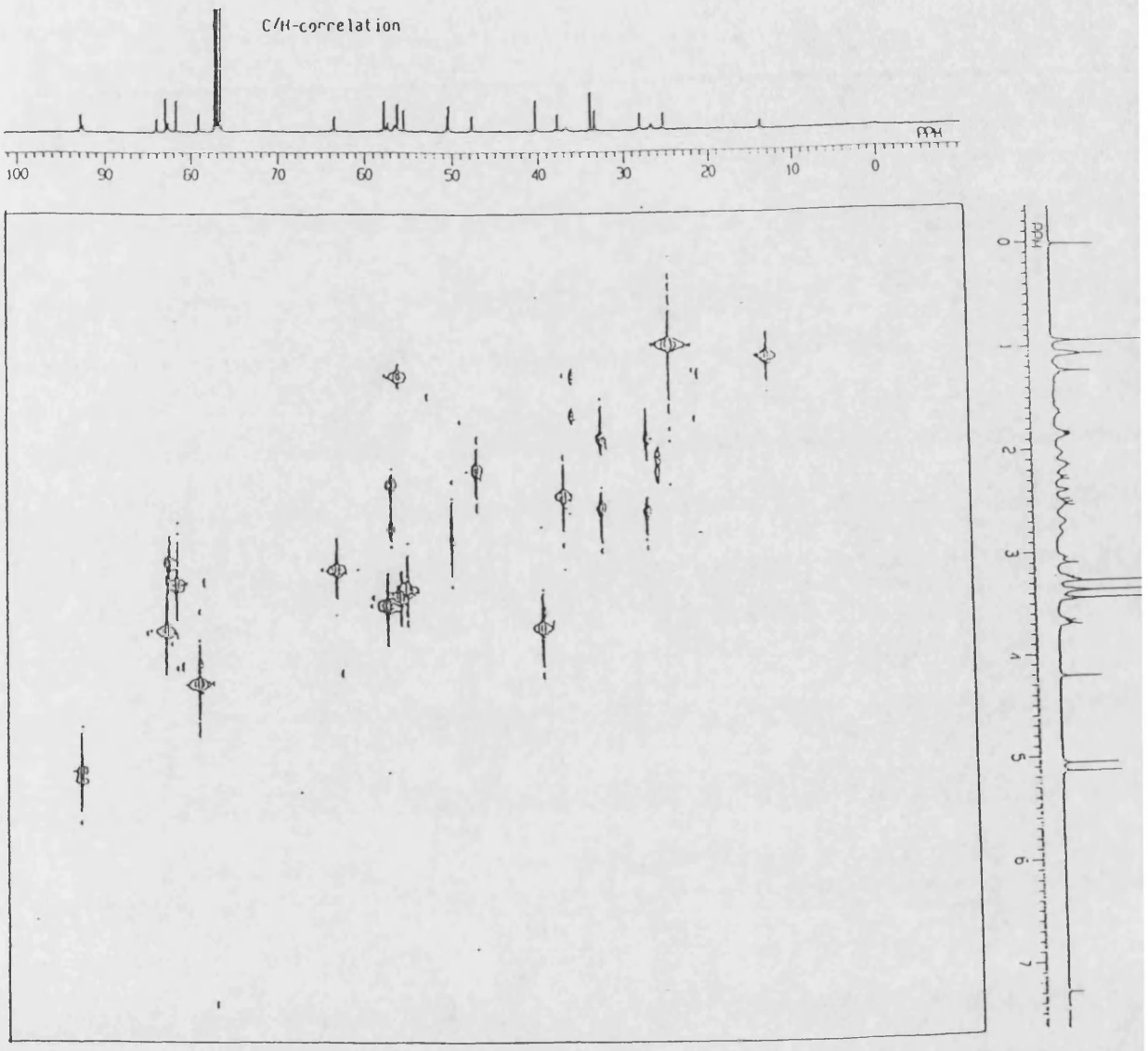


Figure (5) COSY Spectrum of Delpheline (219) in $CDCl_3$

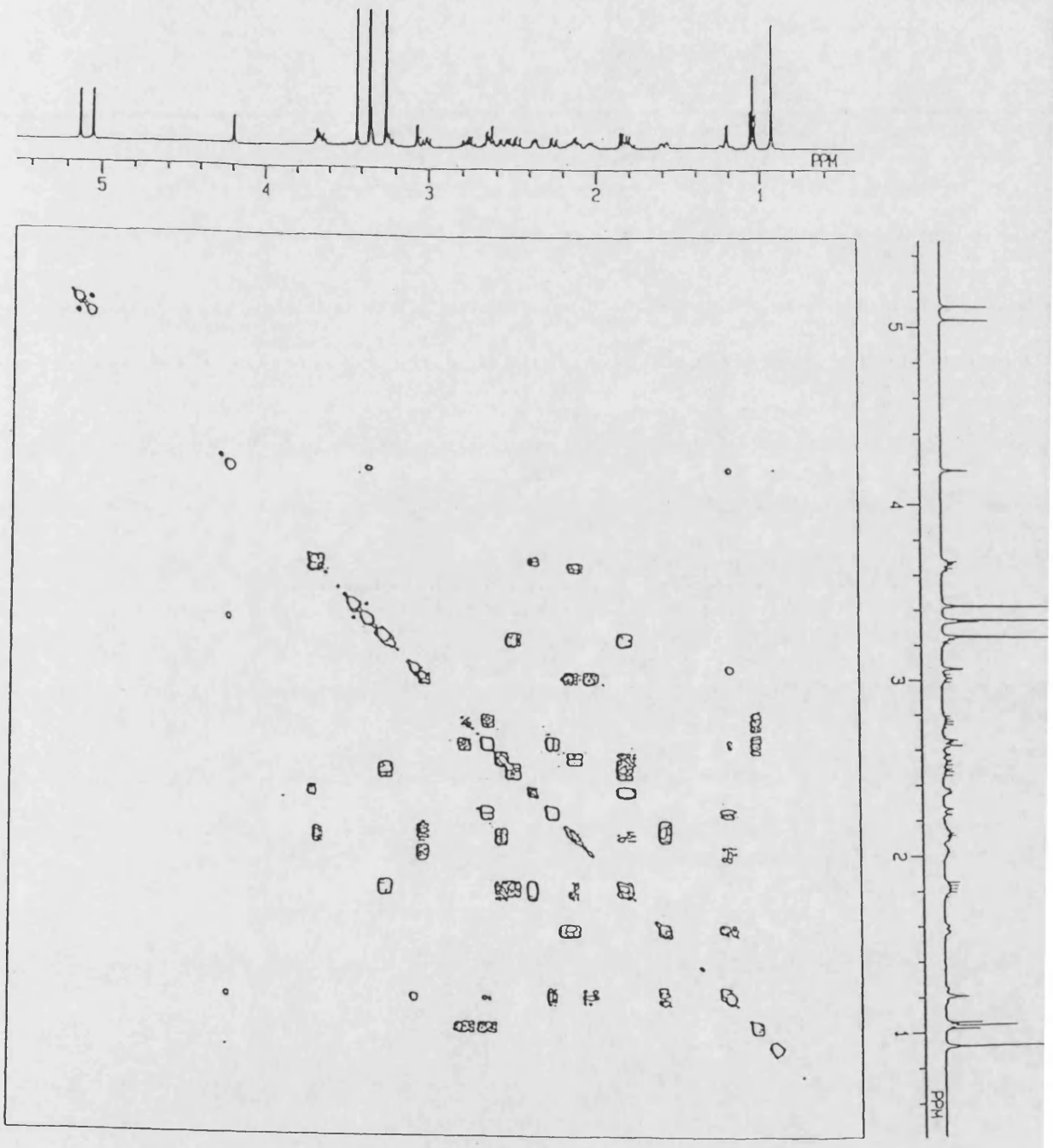


Figure (6) Long-Range COSY Spectrum of Delpheline (219) in $CDCl_3$

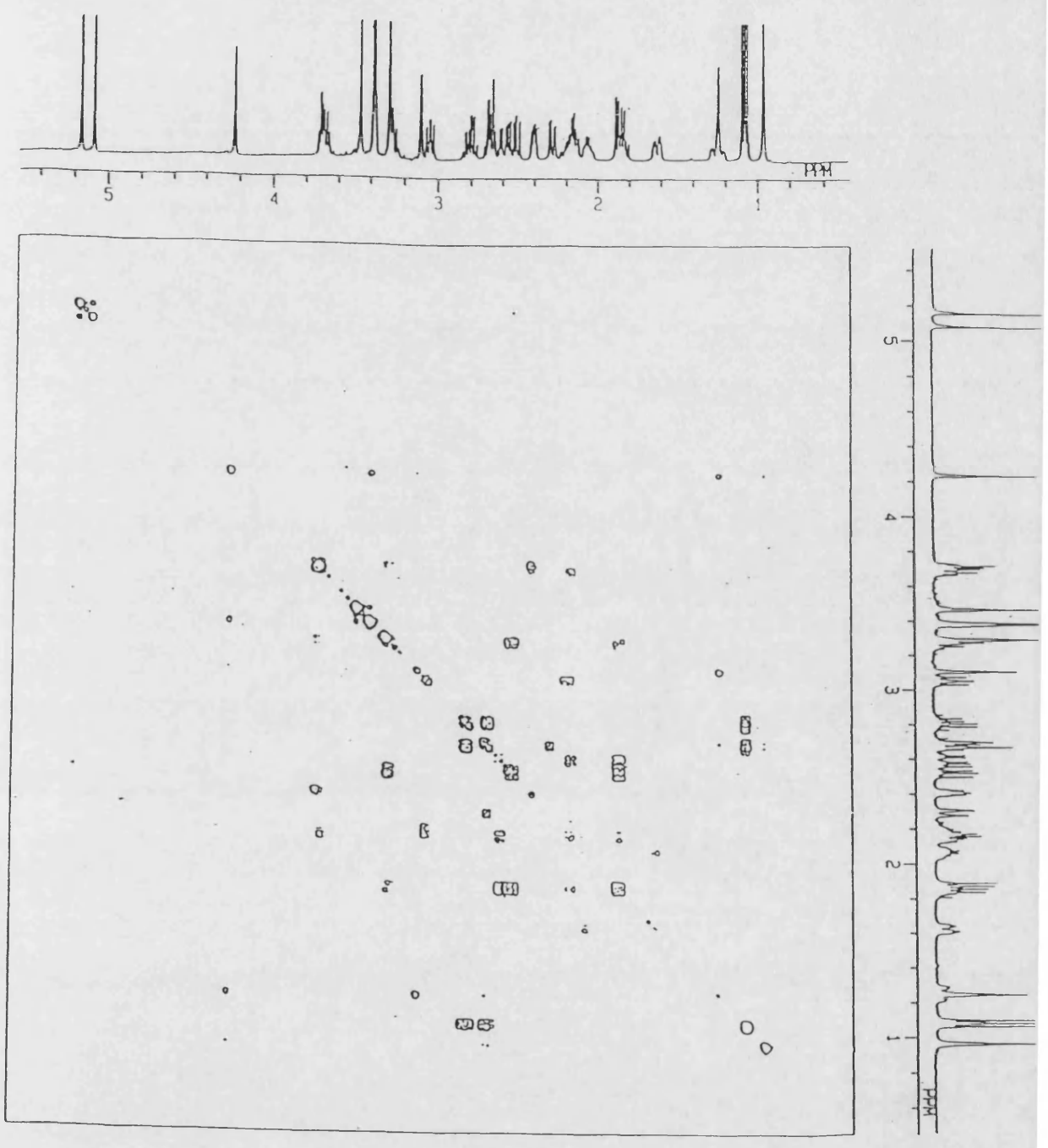


Figure (7) NOESY Spectrum of Delpheline (219) in $CDCl_3$

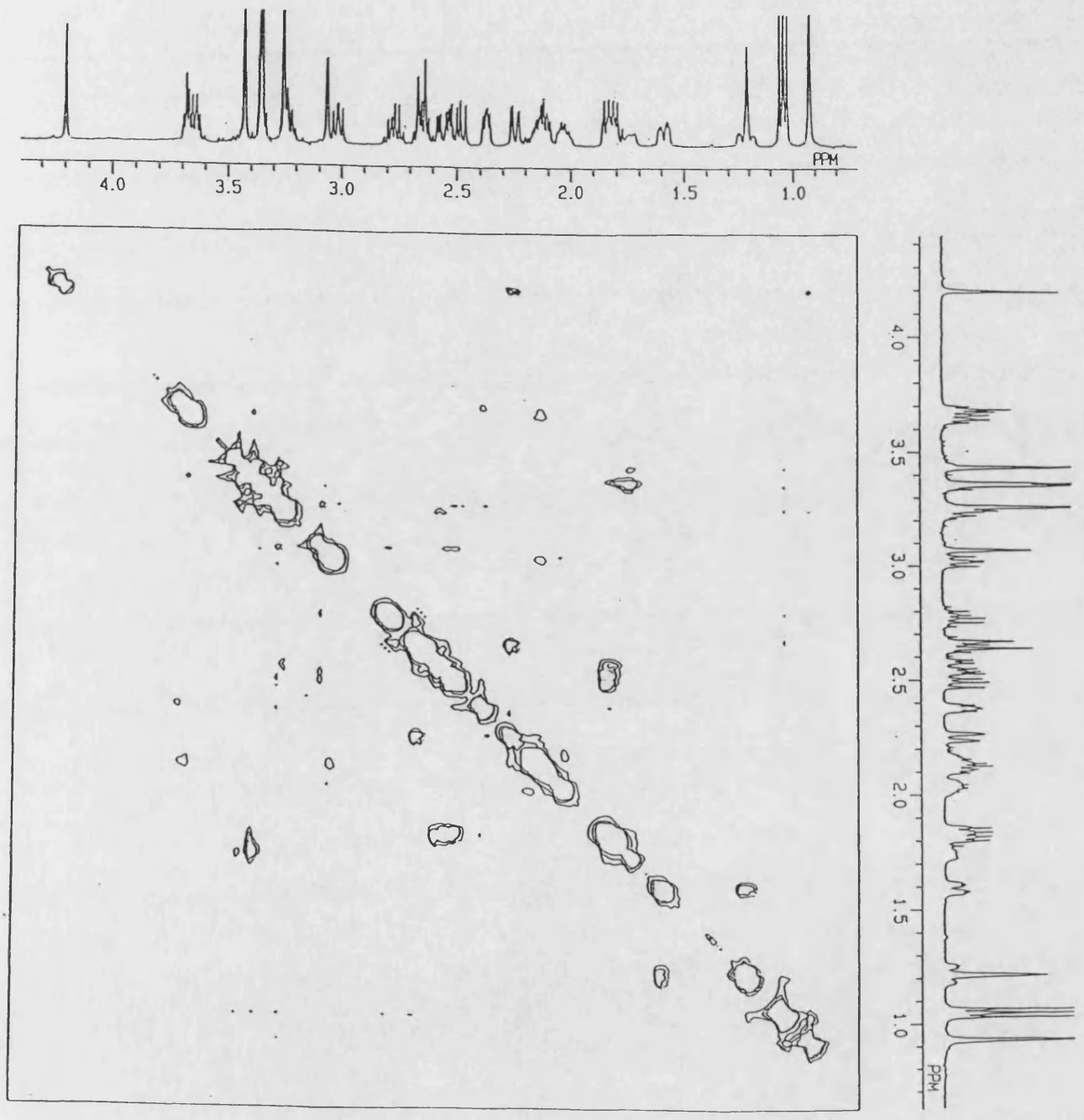


Table 1
NMR Spectral Analysis for Delpheline (219)

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
1	82.7	3.02 (dd, *J* 9.8 and 7.3, H-β-1)	2.07-1.98 (m, H-β-2) 2.21-2.08 (m, H-α-2)	$J_{1\beta2\alpha}$ 9.8 $J_{1\beta2\beta}$ 7.3		1.26-1.16 (m, H-β-3) 2.21-2.08 (m, H-10)
2	26.7	2.07-1.98 (m, H-β-2)	3.02 (dd, H-β-1) 1.26-1.16 (m, H-β-3) 1.59 (ddd, H-α-3)			
		2.21-2.08 (m, H-α-2)	3.02 (dd, H-β-1) 1.59 (ddd, H-α-3)			
3	36.9	1.26-1.16 (m, H-β-3)	2.07-1.98 (m, H-β-2) 1.59 (ddd, H-α-3)		2.24 (d, H-β-19)	3.02 (dd, H-β-1)
		1.59 (ddd, *J* 13.1, 4.9 and 2.1, H-α-3)	2.07-1.98 (m, H-β-2) 2.21-2.08 (m, H-α-2) 1.26-1.16 (m, H-β-3)	$J_{3\alpha3\beta}$ 13.1 $J_{3\alpha2\alpha}$ 4.9 $J_{3\alpha2\beta}$ 2.1		
4	33.8	-				
5	56.6	1.22 (s, H-5)	4.19 (s, H-6)			3.08 (br s, H-17)
6	79.2	4.19 (s, H-6)	1.22 (s, H-5) 3.34 [s, C(6)OH]			0.93 (s, H_3-18) 2.24 (d, H-β-19)
7	92.7	-				
8	84.1	-				
9	40.3	3.67-3.60 (m, H-9)	2.21-2.08 (m, H-10)		2.37 (dd, H-13)	2.37 (dd, H-13) 3.34 [s, C(6)OH]

Table 1 cont.

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
10	47.7	2.21-2.08 (m, H-10)	3.67-3.60 (m, H-9) 1.86-1.78 (m, H-β-12) 2.55 (dd, H-α-12)		3.71-3.64 (m, H-14)	3.02 (dd, H-β-1) 3.71-3.64 (m, H-14)
11	50.4	-				
12	28.1	1.86-1.78 (m, H-β-12)	2.21-2.08 (m, H-10) 2.55 (dd, H-α-12) 2.37 (dd, H-13)			
		2.55 (dd, *J* 14.6 and 4.9, H-α-12)	2.21-2.08 (m, H-10) 1.86-1.78 (m, H-β-12)	$J_{12\alpha 12\alpha}$ 14.6 $J_{12\alpha 10}$ 4.9		3.25-3.19 (m, H-16) 3.26 [s, C(1)OC*H*$_3$]
13	37.7	2.37 (dd, *J* 6.8 and 4.9, H-13)	1.86-1.78 (m, H-β-12) 3.71-3.64 (m, H-14)	$J_{13,14}$ 6.8 $J_{13,12\beta}$ 4.9	3.67-3.60 (m, H-9)	3.67-3.60 (m, H-9)
14	83.0	3.71-3.64 (m, H-14)	2.37 (dd, H-13)		2.21-2.08 (m, H-10) 3.25-3.19 (m, H-16) 3.35 [s, C(16)OC*H*$_3$]	2.21-2.08 (m, H-10)
15	33.4	1.86-1.78 (m, H-β-15)	2.49 (dd, H-α-15) 3.25-3.19 (m, H-16)		5.05 (s, OC*H*α O)	3.25-3.19 (m, H-16)
		2.49 (dd, *J* 14.6 and 8.8, H-α-15)	1.86-1.78 (m, H-β-15) 3.25-3.19 (m, H-16)	$J_{15\alpha 15\beta}$ 14.6 $J_{15\alpha 16}$ 8.8	5.05 (s, OC*H*α O)	3.08 (br s, H-17)
16	81.8	3.25-3.19 (m, H-16)	1.86-1.78 (m, H-β-15) 2.49 (dd, H-α-15)		3.71-3.64 (m, H-14)	2.55 (dd, H-α-12) 1.86-1.78 (m, H-β-15)
17	63.6	3.08 (br s, H-17)			1.22 (s, H-5)	2.49 (dd, H-α-15)
18	25.3	0.93 (s, H_3-18)			4.19 (s, H-6)	
19	57.4	2.24 (d, *J* 11.7, H-β-19)	2.69-2.60 (m, H-α-19)	$J_{19\beta 19\alpha}$ 11.7		1.26-1.16 (m, H-β-3) 4.19 (s, H-6)
		2.69-2.60 (m, H-α-19)	2.24 (d, H-β-19)			

Table 1 cont.

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
NCH_2CH_3	50.6	2.69-2.60 (m, 1 of NCH_2 CH_3)	2.77 (dq, 1 of NCH_2 CH_3) 1.06 (t, NCH_2CH_3)			
		2.77 (dq, *J* 7.1 and 12.0, 1 of NCH_2 CH_3)	2.69-2.60 (m, 1 of NCH_2 CH_3) 1.06 (t, NCH_2CH_3)	$J_{NCH_2CH_3}$ 7.1 J_{NCH_2, NCH_2} 12.0		
NCH_2CH_3	13.8	1.06 (t, *J* 7.1, NCH_2CH_3)	2.69-2.60 (m, 1 of NCH_2 CH_3) 2.77 (dq, 1 of NCH_2 CH_3)	$J_{NCH_2CH_3}$ 7.1		
$C(1)OCH_3$	55.5	3.26 [s, $C(1)OCH_3$]				2.55 (dd, H-α-12)
		3.34 [s, C(6)OH]	4.19 (s, H-6)		5.13 (s, $OCH\beta$ O)	3.67-3.60 (m, H-9)
$C(14)OCH_3$	57.8	3.43 [s, $C(14)OCH_3$]				
$C(16)OCH_3$	56.3	3.35 [s, $C(16)OCH_3$]			3.71-3.64 (m, H-14)	
OCH_2O	92.9	5.05 (s, $OCH\alpha$ O)	5.13 (s, $OCH\beta$ O)		1.86-1.78 (m, H-β-15) 2.49 (dd, H-α-15)	
		5.13 (s, $OCH\beta$ O)	5.05 (s, $OCH\alpha$ O)		3.34 [s, C(6)OH]	

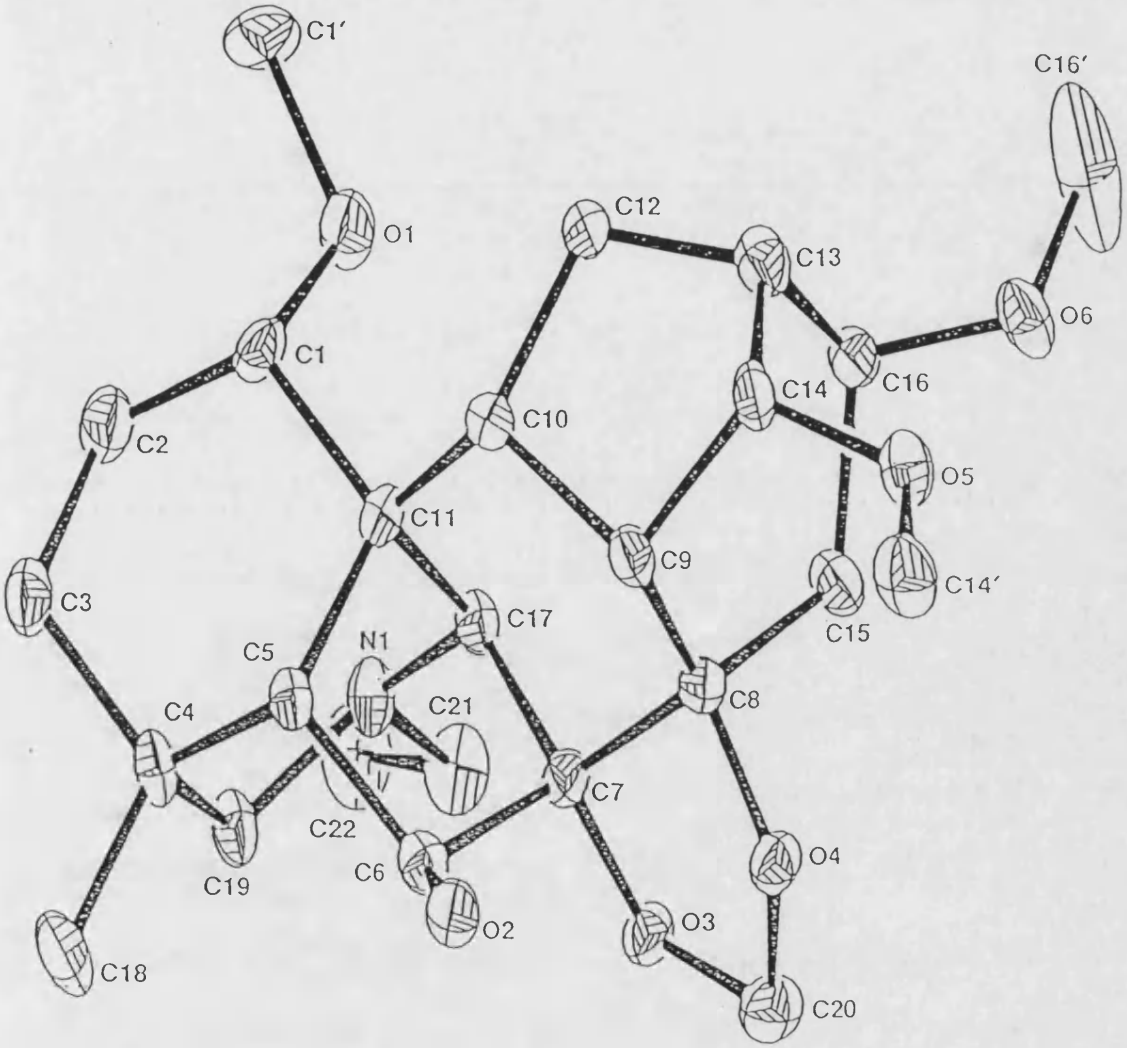
So far, only two complete NMR assignments for a norditerpenoid alkaloid containing 7,8-methylenedioxy substitution, have been published, both for delpheline (219) (Bando *et al.*, 1989 and Joshi *et al.*, 1991). However, these two assignments disagree. Pelletier and colleagues (Joshi *et al.*, 1991) reassigned a number of ^{1}H NMR shifts of Bando and co-workers (1989), and our results are in agreement with those revisions (Joshi *et al.*, 1991). The ^{1}H and ^{13}C NMR spectra of delpheline (219) can be confidently employed, as a template, in the assignment of other 7,8-methylenedioxy substituted C_{19}-diterpenoid alkaloids.

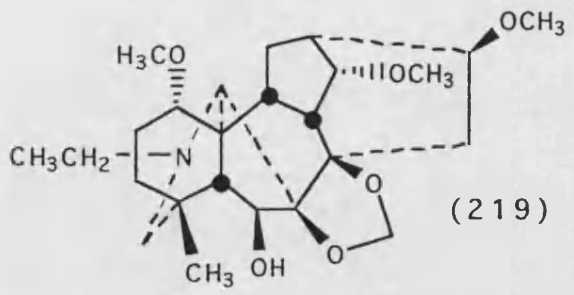
2.2.4 X-Ray Structure of Delpheline

X-ray crystallographic data for delpheline (219) (obtained from a crystal of approximate dimensions 0.3 x 0.3 x 0.4mm) indicated that the orthorhombic crystal belonged to the space group $P2_12_12_1$ (See Section 2.3.2.5 and **Appendix 2**). Figure (8) shows a composite stereoview of delpheline (219) showing the asymmetric unit, along with the labelling scheme used. All hydrogen atoms have been omitted for clarity. Thus, the isolation of the alkaloid delpheline (219) is confirmed and its characterization is complete.

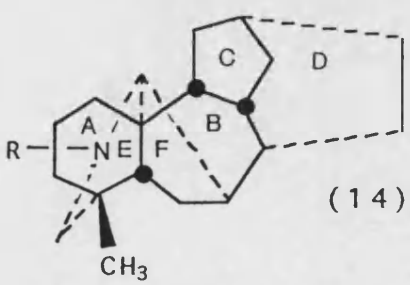
Data to be found in **Appendix 2** includes: anisotropic temperature factors/thermal parameters U ($Å^2$ x10^3), equivalent isotropic thermal parameters U_{eq} ($Å^2$ x10^3), and positional parameters/final fractional atomic co-ordinates (x10^4) for non-hydrogen atoms; Isotropic thermal parameters U ($Å^2$ x10^3) and final fractional atomic co-ordinates (x10^4) for hydrogen atoms; bond distances (Å) and angles (°); selected non-bonded intramolecular and intermolecular distances (Å).

Figure (8) X-Ray of Delpheline (219)

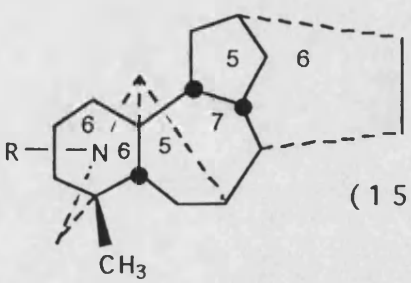




(219)



(14)



(15)

X-ray studies have proved invaluable in establishing the absolute stereochemistry for norditerpenoid alkaloids (Joshi and Pelletier, 1987 and Joshi *et al.*, 1987). The X-ray crystallographic investigations of Edwards and Przbylska (1982) centred on lycoctonine-type alkaloids, but other researchers have studied aconitine (5) and related alkaloids (Codding, 1982).

In our investigations, the relative stereochemistry of the alkaloid at all the centres can be assumed to be correct. Edwards and Przybylska (1982) revised the stereochemistry for all alkaloids related to lycoctonine (2) with a methoxyl group at C-1, such that the β-configuration initially assigned was established as an error. The substitution pattern for delpheline (219) can, therefore, be drawn as C(1)-α-OCH_3, C(6)-β-OH, C(7)-β-OCH_2O-β-C(8), C(14)-α-OCH_3, C(16)-β-OCH_3, *N*-CH_2CH_3 and the absolute configuration considered as: 1*S*, 4*S*, 5*R*, 6*S*, 7*S*, 8*S*, 9*R*, 10*R*, 11*S*, 13*R*, 14*S*, 16*S*, 17*R*.

The central ring system of a norditerpenoid alkaloid is traditionally viewed as being three six-membered (two cyclohexane and one piperidine), two five-membered, and one seven-membered fused rings, but could be viewed as being formed by the fusion of four six-membered and two five-membered fused rings. This inflexible framework only has conformational freedom in ring A and in the free edge of ring D. The ring conformation appears to be determined by H-bond formation. The A/B ring junction is *trans* and all the other ring junctions (A/E, B/C, B/D, and B/F) are *cis*.

Ring A, one of the cyclohexane rings, -C(1)-C(2)-C(3)-C(4)-C(5)-C(11)-, was found to be a chair, with the C-1 substituent [C(1)-α-OCH_3] on the same side of the ring as the *N* bridge. The chair form for ring A has only been found when no opportunity for formation of a hydrogen bond with the *N* atom exists. This occurs when there is no H-atom donor (or for example, a protonated *N* atom and oxygenated function are on opposite sides of the ring), when there is no

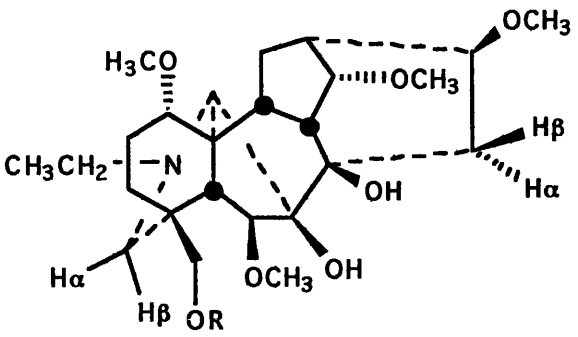
oxygenated function at C-1, and when there is hydrogen-bond formation with a counter ion (Kerr and Codding, 1982 and Codding, 1982). Thus, the chair form is expected for delpheline (219) and is often found for lycoctonine-type and aconitine-type free bases, with α-methoxyl groups at C-1 [for example, lycoctonine (2) (Joshi and Pelletier, 1987), aconitine (5) (Codding, 1982)]. The acetone complex of dictyocarpine (20), an alkaloid possessing the methylene dioxy bridge and the C(1)-α-OCH_3 function found in delpheline, was similarly found to have ring A in a chair conformation (Joshi and Pelletier, 1987). In the chair form, the *N* atom is exposed and this may effect the interaction with the receptor site (Codding, 1982). At biological pH, however, the *N* atom of such alkaloids may be protonated (Codding, 1982), permitting the formation of a hydrogen bond from the ammonium hydrogen to the oxygen atom of the group at C-1 and thus, stabilizing the boat form (C-2 located *cis* rather than *trans* to C-5 with reference to the plane passing through C-1, C-3, C-4, and C-11) (Codding, 1982). A boat form is often found for this six-membered ring in the salts of norditerpenoid alkaloids [for example, the perchlorate of browniine (Joshi and Pelletier, 1987)] due to a hydrogen bond between the *N* atom and the counter ion (Codding, 1982). In addition, norditerpenoid alkaloids bearing a C(1)-α-OH, such as delphinifoline or delvestine (46), usually exist with ring A in a boat conformation to facilitate the intramolecular hydrogen bonding between the nitrogen and hydroxyl group, either with the hydroxyl oxygen accepting a proton from *N* or with an unprotonated *N* atom interacting with the hydroxyl hydrogen (Kerr and Codding, 1982 and Bhandary *et al.*, 1990, respectively). Kerr and Codding (1982) suggested that the energy barrier between boat and chair forms is relatively low for ring A in these compounds.

The six-membered ring, ring D, -C(8)-C(9)-C(14)-C(13)-C(16)-C(15)-, does not have the flexibility of ring A. It is in half-chair form with C-14 and C-15 forming the end atoms above the plane through C-8, C-9, C-13, and C-16. For alkaloids possessing C-7, C-8 dihydroxy substitution, as in many other lycoctonine-type norditerpenoid alkaloids, a boat (with the end at C-15 flattened) is possible,

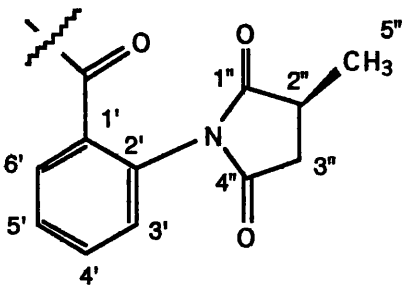
stabilized by a bifurcated intramolecular hydrogen bond between the hydrogen of C(7)-β-OH and the oxygen of C(8)-β-OH (Kerr and Codding, 1982).
The ring -C(7)-C(8)-C(9)-C(10)-C(11)-C(17)- in delpheline (219) is a distorted chair with C-9 below and C-17 above the plane through the remaining four atoms, whereas, in acoforestine, the atoms C-7 and C-10 deviate from the plane (Bhandary *et al.*, 1990). The piperidine ring, ring E, -C(4)-C(5)-C(11)-C(17)-N-C(19)-, is also a distorted chair, with *N* above and C-5 below the plane through the atoms C-4, C-11, C-17, and C-19. Five-membered ring C, -C(9)-C(10)-C(12)-C(13)-C(14)-, is in an envelope conformation, with C-14 at the flap. Ring B, the seven-membered ring, -C(5)-C(6)-C(7)-C(8)-C(9)-C(10)-C(11)-, is a chair, with the C-10 and C-6 atoms deviating from the plane, as seen for aconitine (5) (Codding, 1982). Ring F, one of the five-membered rings, -C(5)-C(6)-C(7)-C(17)-C(11)-, is puckered. For some alkaloids, for example aconitine (5), it is possible to describe this ring as a half-chair with an approximate two-fold axis through C-6. As an illustration of the effect of the substituents around the norditerpenoid skeleton, delcosine (19), which differs from delpheline (219) in that it has hydroxyl groups at C-1, C-7, C-8, and C-14 as well as C-6 and a methoxyl group at C-18, has rings A, B, and D in boat conformations, rings C and F in envelope form, and ring E as a chair (Joshi and Pelletier, 1987).

2.2.5 NMR Assignment of MLA

The middle band (Fraction 2) (purified by any one of the three afore mentioned techniques) appeared to contain one major component with r_f = 0.30 (cyclohexane-chloroform-diethylamine), the same as an authentic sample of MLA, obtained by treatment of MLA. citrate with alkali. A comprehensive set of spectroscopic data (See Section 2.3.2.6) was again obtained for and the non-cystallizable foam was confirmed to be the important alkaloid, MLA (1). The ^1H NMR spectrum (400MHz) and ^{13}C NMR spectra (100.4MHz) are shown in Figures (9) and (10) and Figures (11)-(13) show HETCOR, COSY and COLOC spectra (NOESY and long-range COSY spectra were also obtained but are not shown here).



R =

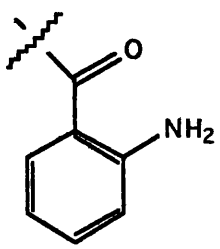


(1) Natural MLA

(250) Semi-Synthetic MLA

R = H (2) Lycoctonine

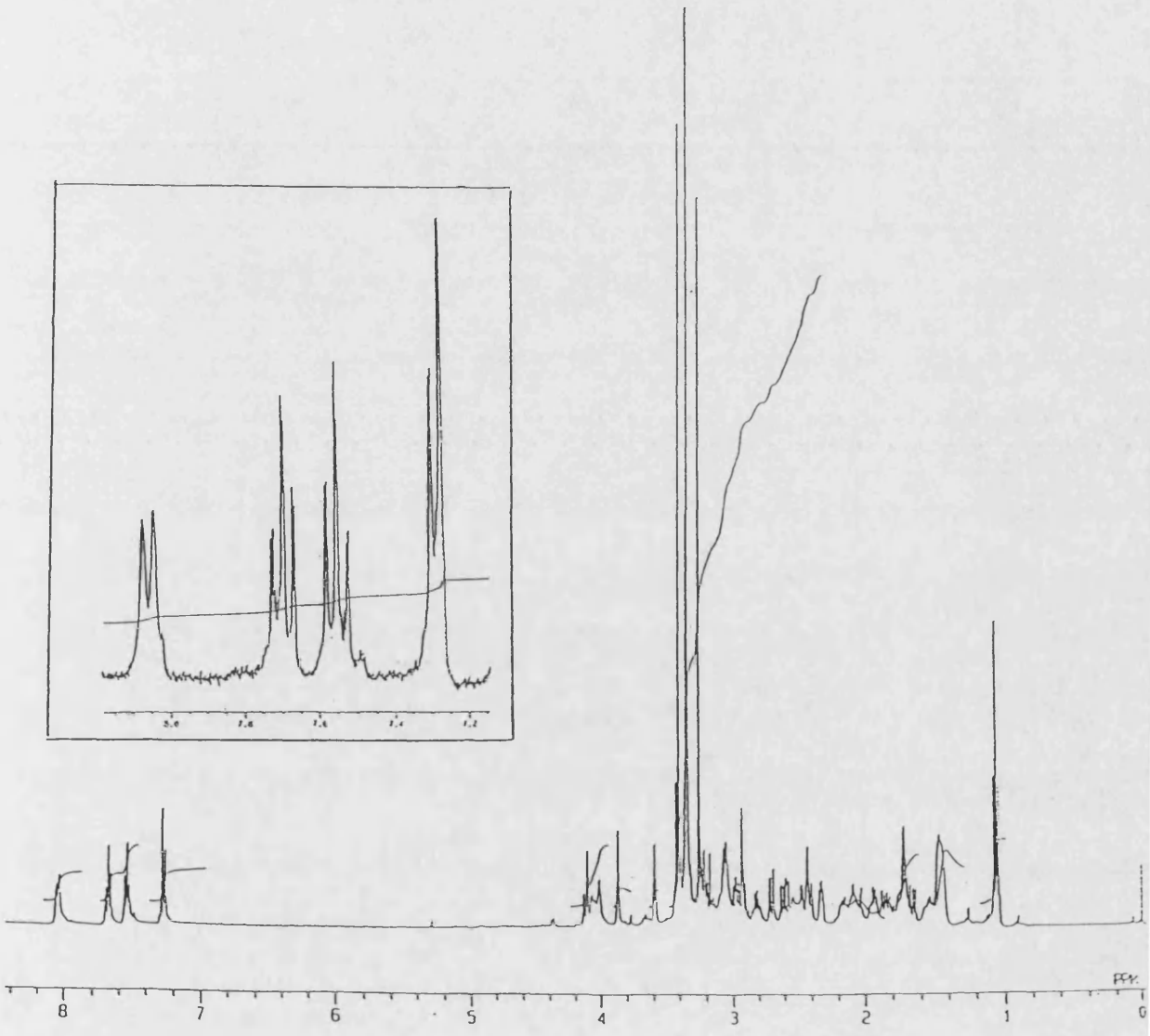
R =



(40) Inuline

Figure (9) ^1H NMR (400MHz) Spectrum of MLA (1) in $CDCl_3$

Inset: Expansion of the Aromatic Region



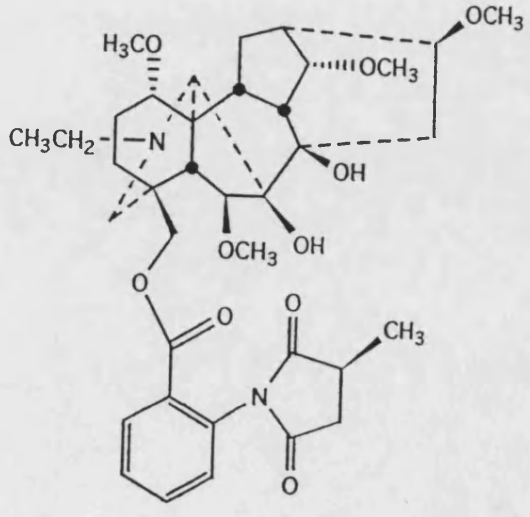
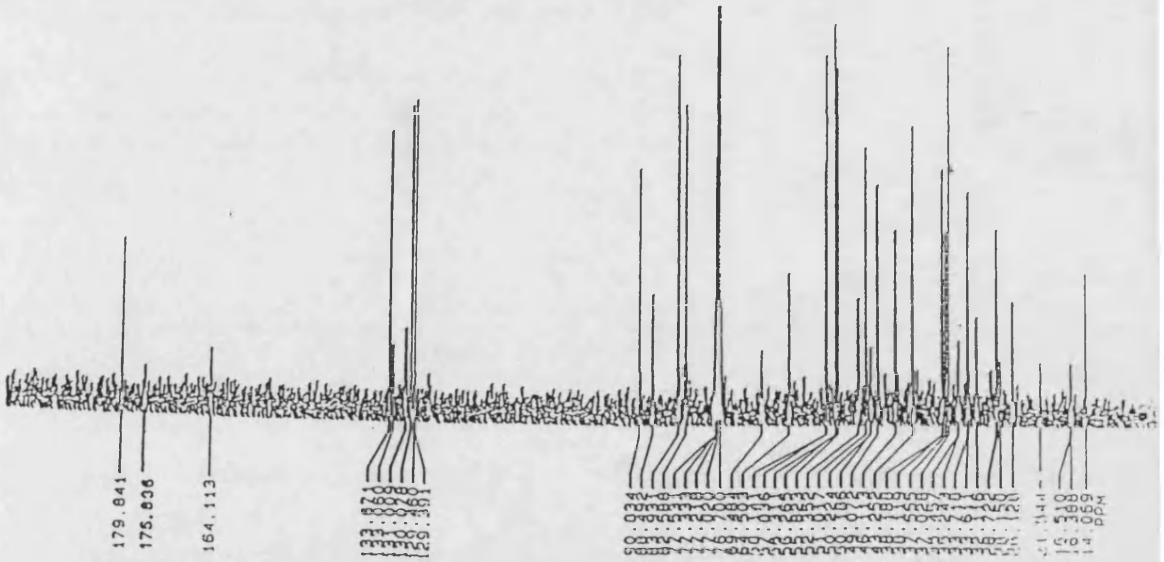


Figure (10) ^{13}C NMR (100.4MHz) Spectrum of MLA (1) in $CDCl_3$



In **Table 2** all the NMR spectral analysis for MLA (1) can be found and in Section 2.2.8 comparison of the data with the other alkaloids under investigation is made. Our spectra show that H_2-18 displays some AB character.

As seen for delpheline (219), the diagnostic region for the methoxy signals overlapped the C-19 signal which is split in the HETCOR spectrum [Figure (11)] to reflect correlation with the two different proton environments for H_2-19, α and β. Also overlapping, in this region of the ^{13}C NMR spectrum [Figure (10)], is the C-5 signal (COLOC spectrum offers no information on this occasion).

The C(1)-methoxyl protons are located by the coupling of C(1)-α-OCH_3 to H-α-3 in the COSY spectrum [Figure (12)]. The COLOC spectrum [Figure (13)] was important in assigning the methoxyl groups, revealing crosspeaks connecting C-16 to C(16)OCH_3 , C-6 to C(6)OCH_3 , C(6)OCH_3 to H-6, and C-11 to C(1)-α-OCH_3 . By elimination, therefore, C(14)OCH_3 can be assigned. The hydroxyl groups could not be assigned, even by deuterium exchange, possibly because of heavy overlapping peaks.

Six of the signals in the ^{13}C and 135° and 90° DEPT ^{13}C spectra (not shown) were in the aromatic region of the spectrum and could be correlated to the

corresponding four protons of the anthranoyl ester group in the HETCOR spectrum. Another assignment of particular significance in the ^{13}C NMR spectra [Figure (10)] is 164.1 (C=O) (Pelletier *et al.*, 1981b). The ^{1}H NMR spectrum [Figure (9)] showed a pattern, consisting of two triplets (dt) and two doublets (dd) in the aromatic region, as might have been expected. The *meta* coupling constants, J = 1.6Hz and J = 1.3Hz, from the expansion of the ^{1}H NMR spectrum at 400MHz (spectrum not shown) were successfully picked out. In the ^{1}H-^{1}H correlated spectrum [Figure (12)] 3 strong *ortho* cross-spots and 2 weaker ones for the *meta* interactions (change of threshold), were seen. The most downfield proton would be expected to be the one bonded to the carbon adjacent to the carbon attached to the carbonyl, that is, H-6'. Hence it was possible to assign all protons {and therefore, carbons using the HETCOR spectrum [Figure (11)]} and associated coupling constants. The proton assignments agree with the trend for other 2-aminobenzoate esters and (*N*-acetyl) 2-aminobenzoate esters but, to date, these assignments for our alkaloid have not been reported (Desai *et al.*, 1994 and Sayed *et al.*, 1992). The ^{13}C NMR assignments reported here with confidence are not in agreement with the literature (Chen and Wu, 1990 and Sun and Benn, 1992).

The crucial region of δ1.5ppm in the ^{1}H NMR spectrum of the aromatic containing alkaloid [Figure (9)], where the signal for the methyl group of the imide ring is expected, revealed an extremely broad signal (doublet, $J_{5"2"}$ = 7.8Hz) at δ1.47ppm so further supporting the evidence for the presence of MLA [J = 6Hz (Pelletier *et al.*, 1981b)]. In the literature, there is some controversy as to the assignment of the carbons of the methylsuccinimido group (Pelletier *et al.*, 1977, 1981b and 1984 Sun and Benn, 1992 and Chen and Wu, 1990). This seems extraordinary due to the clear indication given by the DEPT as to whether a carbon is a quaternary, methine, methylene, or methyl carbon. In the ^{13}C NMR spectra [Figure (10)] observe 179.8 (C-1"), 37.0 (C-3"), 35.2 (C-2"), and 16.4ppm (C-5"). Geminal cross-spots are observed in the COSY spectrum [Figure (12)] and both H-3" s to H-2" and in addition, some coupling to H-5" is seen in the ordinary and long-range COSY spectra).

Figure (11) HETCOR Spectrum of MLA (1) in $CDCl_3$

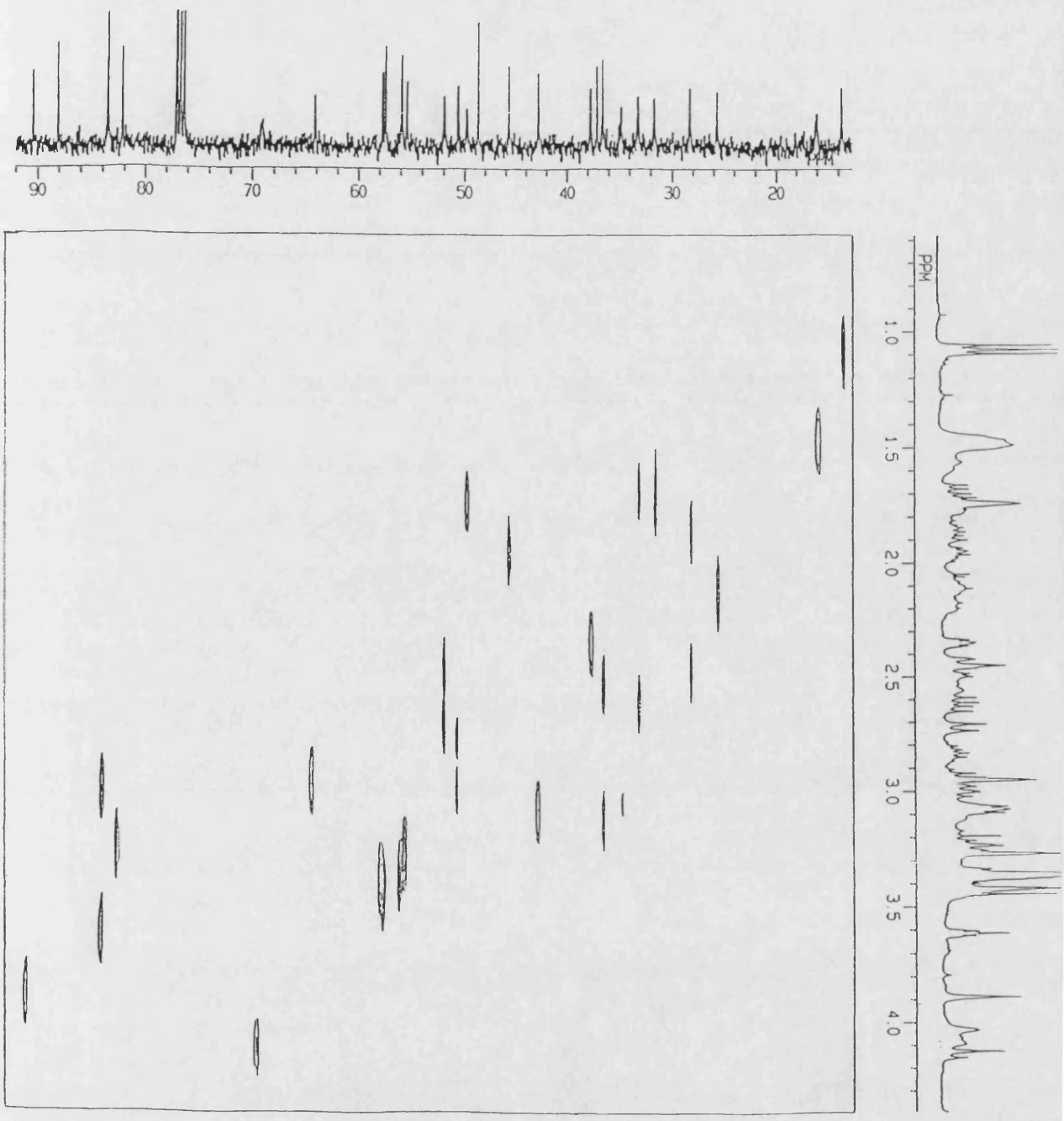


Figure (12) COSY Spectrum of MLA (1) in $CDCl_3$

Inset: Expansion of the Aromatic Region

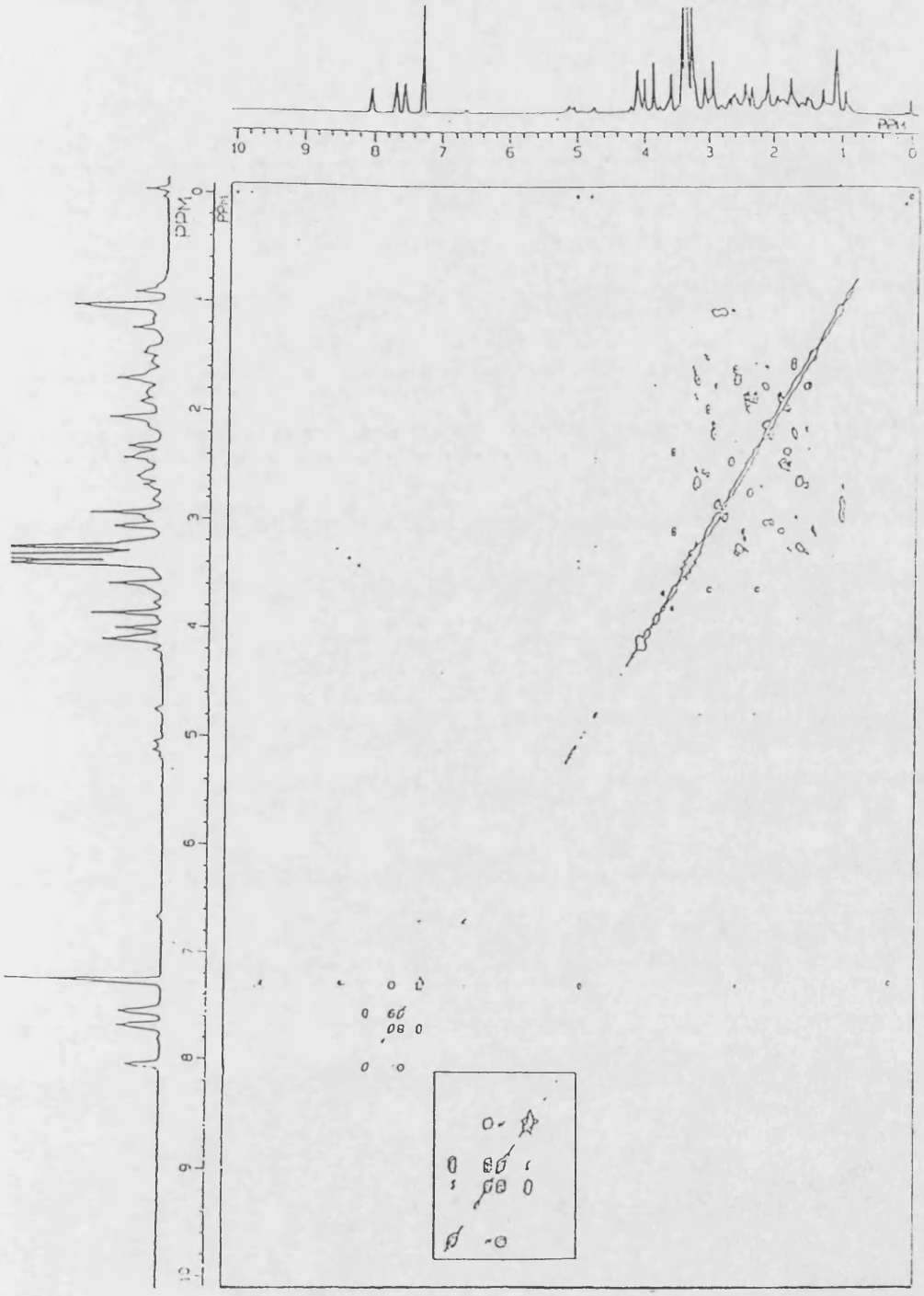


Figure (13) COLOC Spectrum of MLA (1)

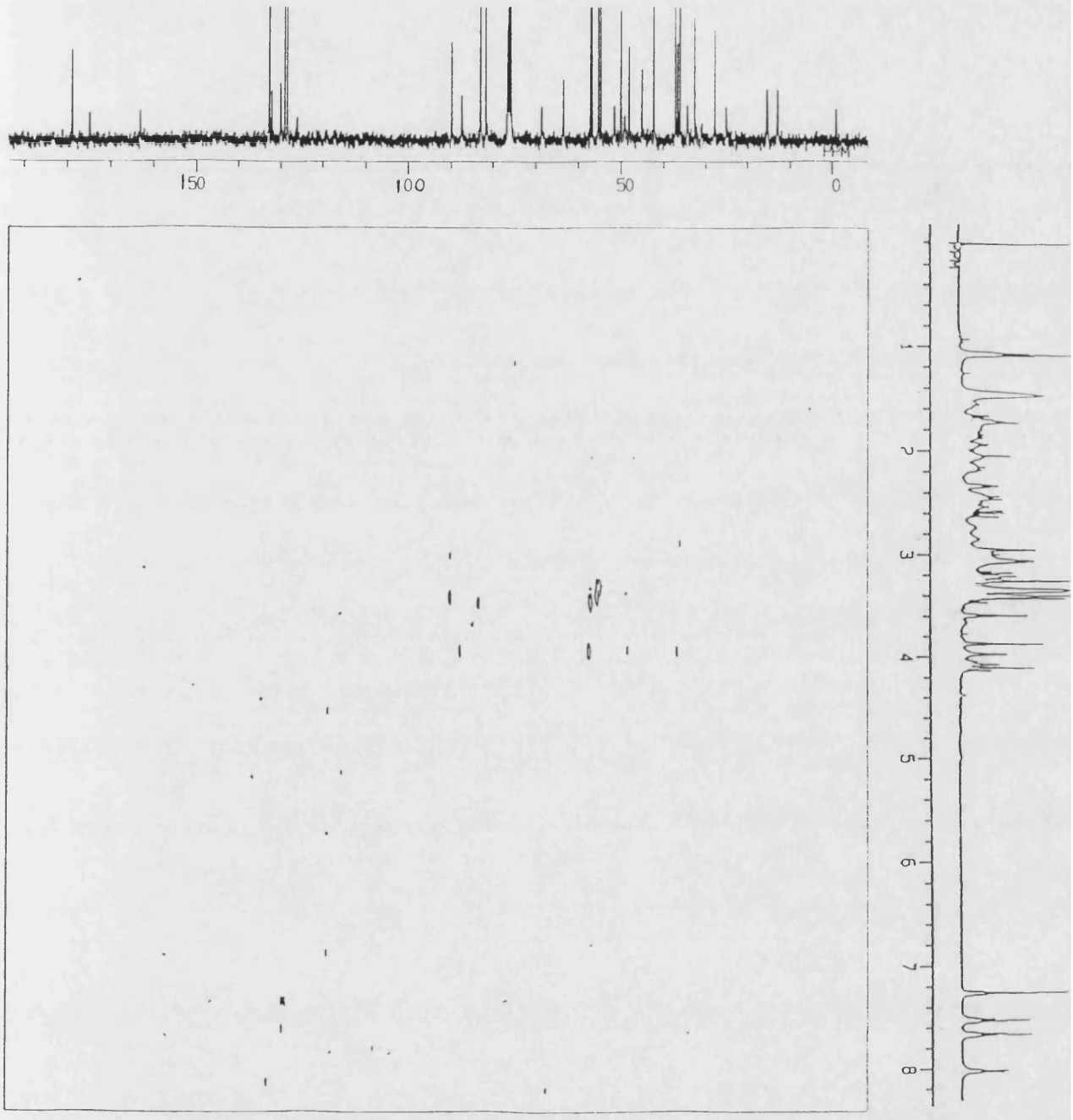


Table 2
NMR Spectral Analysis for MLA (1)

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
1	83.9	2.95-2.92 (m, H-β-1)	2.15-2.03 (m, H-β-2) 2.20-2.15 (m, H-α-2)		1.78-1.72 (m, H-α-3)	
2	26.1	2.15-2.03 (m, H-β-2)	2.95-2.92 (m, H-β-1) 2.20-2.15 (m, H-α-2) 1.58-1.52 (m, H-β-3) 1.78-1.72 (m, H-α-3)			
		2.20-2.15 (m, H-α-2)	2.95-2.92 (m, H-β-1) 2.15-2.03 (m, H-β-2) 1.78-1.72 (m, H-α-3)			
3	32.1	1.58-1.52 (m, H-β-3)	2.15-2.03 (m, H-β-2) 1.78-1.72 (m, H-α-3)			
		1.78-1.72 (m, H-α-3)	2.15-2.03 (m, H-β-2) 2.20-2.15 (m, H-α-2) 1.58-1.52 (m, H-β-3)		2.95-2.92 (m, H-β-1) 3.85 (s, H-6)	3.28 [s, C(1)OC*H*$_3$]
4	37.6	-				
5	50.3	1.70-1.64 (m, H-5)	3.85 (s, H-6)		2.95-2.92 (m, H-17)	2.45-2.38 (m, H-β-19)
6	90.8	3.85 (s, H-6)	1.70-1.64 (m, H-5)		1.78-1.72 (m, H-α-3)	
7	88.5	-				
8	77.5	-				
9	43.2	3.10-3.03 (m, H-9)	1.98-1.90 (m, H-10) 3.60 (dd, H-14)			

Table 2 cont.

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
10	46.1	1.98-1.90 (m, H-10)	3.10-3.03 (m, H-9) 1.90-1.80 (m, H-β-12)			
11	49.1	-				
12	28.7	1.90-1.80 (m, H-β-12)	1.98-1.90 (m, H-10) 2.50-2.45 (m, H-α-12) 2.35-2.31 (m, H-13)		3.24-3.17 (m, H-16)	
		2.50-2.45 (m, H-α-12)	1.90-1.80 (m, H-β-12)		3.24-3.17 (m, H-16)	
13	38.0	2.35-2.31 (m, H-13)	1.90-1.80 (m, H-β-12) 3.60 (dd, H-14)			
14	83.0	3.60 (dd, *J* 4.8 and 4.6, H-14)	3.10-3.03 (m, H-9) 2.35-2.31 (m, H-13)	$J_{14,13}$ 4.8 $J_{14,9}$ 4.6		
15	33.6	1.70-1.64 (m, H-β-15)	2.64-2.56 (m, H-α-15) 3.24-3.17 (m, H-16)			
		2.64-2.56 (m, H-α-15)	1.70-1.64 (m, H-β-15) 3.24-3.17 (m, H-16)			
16	82.6	3.24-3.17 (m, H-16)	1.70-1.64 (m, H-β-15) 2.64-2.56 (m, H-α-15)		1.90-1.80 (m, H-β-12) 2.50-2.45 (m, H-α-12)	
17	64.5	2.95-2.92 (m, H-17)			1.70-1.64 (m, H-5)	
18	69.5	4.15-4.00 (m, H-β-18)	4.15-4.00 (m, H-α-18)			
		4.15-4.00 (m, H-α-18)	4.15-4.00 (m, H-β-18)			
19	52.4	2.45-2.38 (m, H-β-19)				2.73-2.69 (m, H-α-19)
		2.73-2.69 (m, H-α-19)			1.70-1.64 (m, H-5)	2.45-2.38 (m, H-β-19)

Table 2 cont.

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
NCH_2CH_3	50.9	2.73-2.69 (m, 1 of NCH_2 CH_3)	2.95-2.92 (m, 1 of NCH_2 CH_3) 1.07 (t, NCH_2CH_3)			
		2.95-2.92 (m, 1 of NCH_2 CH_3)	2.73-2.69 (m, 1 of NCH_2 CH_3) 1.07 (t, NCH_2CH_3)			
NCH_2CH_3	14.1	1.07 (t, J 7.0, NCH_2CH_3)	2.73-2.69 (m, 1 of NCH_2 CH_3) 2.95-2.92 (m, 1 of NCH_2 CH_3)	$J_{NCH_2CH_3}$ 7.0		
$C(1)OCH_3$	55.8	3.28 [s, $C(1)OCH_3$]				1.78-1.72 (m, H-α-3)
$C(6)OCH_3$	58.1	3.45 [s, $C(6)OCH_3$]				
		[s, C(7)OH]				
		[s, C(8)OH]				
$C(14)OCH_3$	57.8	3.42 [s, $C(14)OCH_3$]				
$C(16)OCH_3$	56.3	3.38 [s, $C(16)OCH_3$]				

Table 2 cont.

Carbon	δ (ppm)	Correlated Protons					
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [NOESY]
C=O	164.1	-					
1'	126.9	-					
2'	133.1	-					
3'	130.0	7.30 (dd, *J* 7.6 and 1.3, H-3')	7.70 (t, H-4')	$J_{3'4'}$ 7.6	7.55 (t, H-5')	$J_{3'5'}$ 1.3	
4'	133.7	7.70 (dt, *J* 7.6 and 1.6, H-4')	7.30 (d, H-3') 7.55 (t, H-5')	$J_{4'3'}$ 7.6 $J_{4'5'}$ 7.6	8.05 (d, H-6')	$J_{4'6'}$ 1.6	
5'	129.4	7.55 (dt, *J* 7.6 and 1.3, H-5')	7.70 (t, H-4') 8.05 (d, H-6')	$J_{5'4'}$ 7.6 $J_{5'6'}$ 7.6	7.30 (d, H-3')	$J_{5'3'}$ 1.3	
6'	131.0	8.05 (dd, *J* 7.6 and 1.6, H-6')	7.55 (t, H-5')	$J_{6'5'}$ 7.6	7.70 (t, H-4')	$J_{6'4'}$ 1.6	
1"	179.8	-					
2"	35.2	3.02-2.96 (m, H-2")	1.49-1.42 (m, H_3-5")		3.10-3.03 (m, H-3") 2.56-2.50 (m, H-3")		
3"	37.0	2.56-2.50 (m, H-3")	3.10-3.03 (m, H-3")		3.02-2.96 (m, H-2")		
		3.10-3.03 (m, H-3")	2.56-2.50 (m, H-3")		3.02-2.96 (m, H-2")		
4"	175.8	-					
5"	16.4	1.47 (br d, *J* 7.8, H_3-5")	3.02-2.96 (m, H-2")	$J_{5"2"}$ 7.8			

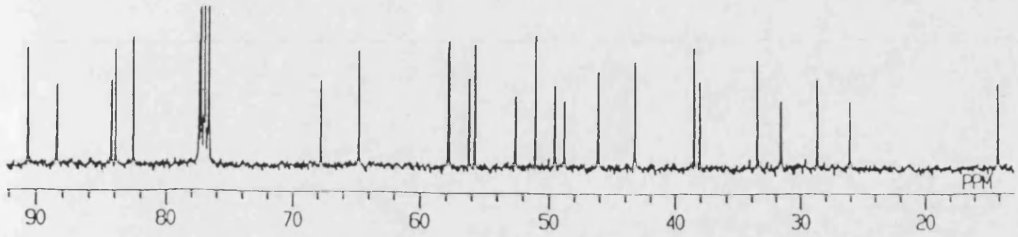
For ring A, an A_2B_2X spin system is observed where X is H-β-1, A_2 is H_2-3, and B_2 is H_2-2. The ^1H-^1H COSY spectrum [Figure (12)] was helpful, revealing crosspeaks between H-β-1 and H-α-2, H-β-1 and H-β-2, H-β-1 and H-α-3. Other couplings seen in the COSY spectrum [Figure (12)] connect H-α-2 to H-β-2, H-α-2 to H-α-3, H-β-2 to H-β-3, H-β-2 to H-α-3, and possibly H-α-3 to H-β-3 (two and three bond couplings). Other couplings seen were NC*H*$_2$ CH_3 or H-α-19 to H-α-2. C-3 was assigned by elimination as 32.1ppm over C-15, using the COLOC spectrum [Figure (13)] in conjunction with the HETCOR spectrum, since H-β-15 couples to both C-9 and C-15. For ring B, an AX pattern is observed. For ring C, an ABX system of H-13 (B), H-9 (A), and H-14 (X) are observed. The couplings observed include H-9 to H-14 and H-13 to H-14, but not H-9 to H-13. On inspection of the fine structure of the expansion of the 1-D proton spectrum (not shown), it is possible to see $J_{13,14}$ = 4.8Hz and $J_{9,14}$ = 4.6Hz, such that the signal for H-14 is a clear doublet of doublets. Crosspeaks in the COSY spectrum [Figure (12)] provided evidence for a *cis* ring junction at C-9 to C-10. An ABC system is caused by H_2-12 (A and C) and H-10 (B). The COLOC spectrum [Figure (13)] is very informative in allowing the assignment of C-10 and C-13 (based on the coupling of C-10 to H-13 and/or C-16 to H-13). Crosspeaks in the COSY spectrum [Figure (12)] confirmed the assignment and stereochemistry of H-β-12 and H-β-13. For ring D, an ABX system is exhibited for H_2-15 (A and B) (a coupling for H-α-15 to H-β-15 is observed) and H-16 (X) (couplings between H-α-15 and H-16 plus H-β-15 to H-16 are observed) [COSY spectrum, Figure (12)]. Other couplings seen were H-α-12 to H-16 (also a very weak one for H-β-12 to H-16). For ring E, C-17 and C-19 are bridged by a nitrogen atom resulting in relatively downfield resonances of H-17 and H_2-19. The only interaction which can be clearly identified is for H-α-19 to H-β-19 in the NOESY spectrum. H_2-19 are observed at δ2.45-2.38ppm and δ2.73-2.69ppm showing some AB character. The methylene protons of the *N*-ethyl side-chain appear as a multiplet at δ2.95-2.69ppm rather than displaying a clear AB system. The terminal methyl group appears as a triplet at δ1.07ppm (NC*H*$_2$ CH_3 coupling to NCH_2CH_3 as expected).

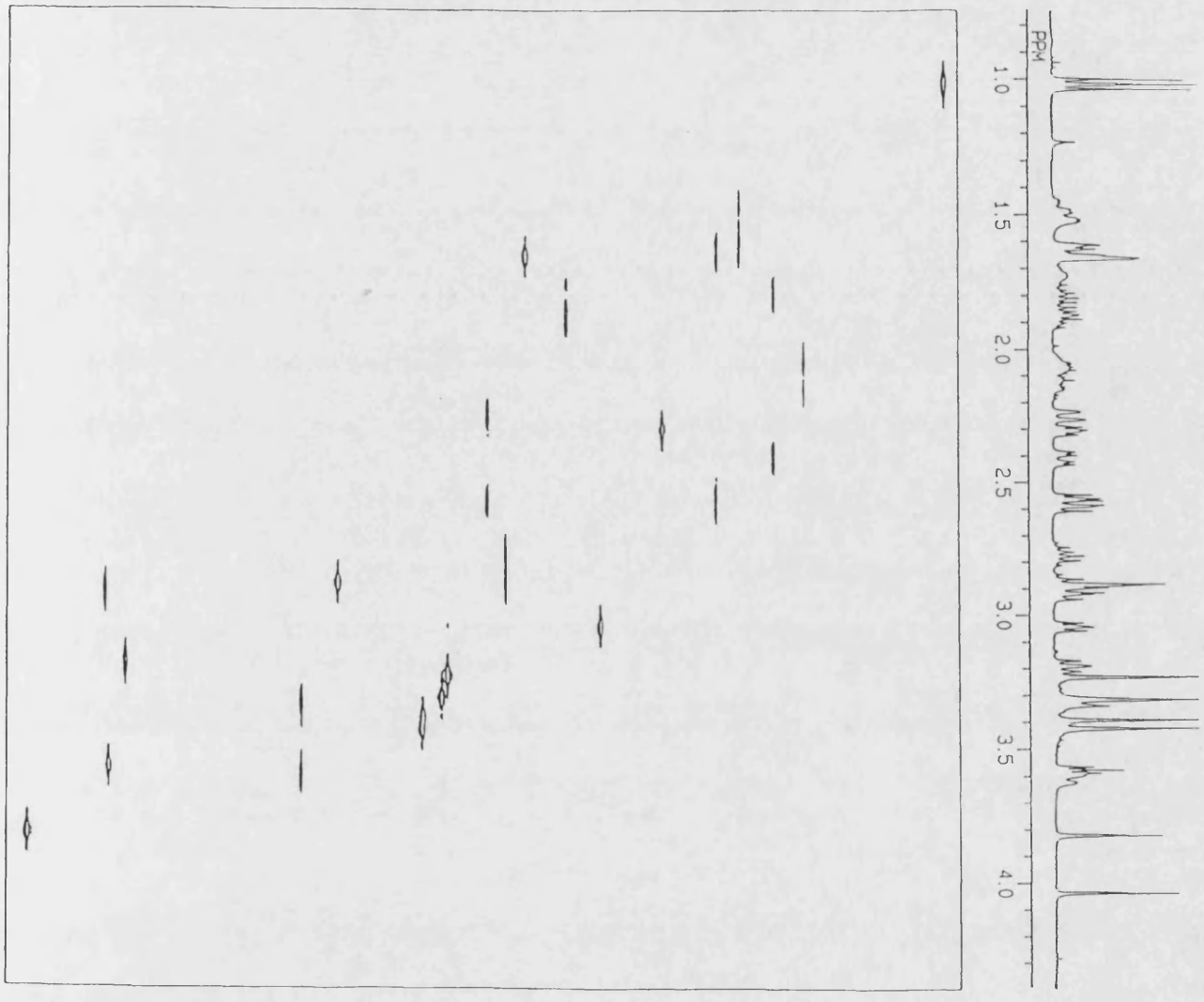
2.2.6 NMR Assignment of Lycoctonine

Hydrolysis of MLA (1) with aqueous sodium hydroxide solution gave the parent alcohol lycoctonine (2) and the *N*-(methylsuccinyl)anthranilic acids (221a) and (221b), as a mixture of isomers (Manske, 1938) (See later in Section 2.2.11 and Section 2.3.2.7). On obtaining alcohol (2) in crystalline form, a similar assignment strategy was followed for norditerpenoid skeleton and the substituents, that is detailed inspection of the 2D NMR spectra, whereby the HETCOR, COSY and phase-sensitive NOESY are to be found on Figures (14), (15) and (16) (1D spectra and long-range COSY and COLOC spectra are available but not shown here). Comparison of our data was made with literature values (Pelletier *et al.*, 1981b, Pelletier *et al.*, 1977, Pelletier *et al.*, 1984, Jones and Benn, 1973, Chen and Wu, 1990). **Table 1** shows the information obtained from the spectra and Section 2.2.8 discusses the data for lycoctonine relative to the other alkaloids in this study (Hanuman and Katz, 1994).

Couplings for each of the relevant methine protons to the associated methoxyl protons were seen along with cross-spots connecting C(1)-α-OCH_3 to H-α-3 and C(16)-β-OCH_3 to H-β-14 [COSY spectrum/long-range COSY spectrum], thus allowing confident assignment of the methoxyl protons. NOESY spectrum [Figure (16)] revealed an interaction between H-β-9 and C(6)-β-OCH_3 and although not evident from CPK model, H-α-6 and C(16)-β-OCH_3 .

Figure (14) HETCOR Spectrum of Lycoctonine (2) in $CDCl_3$





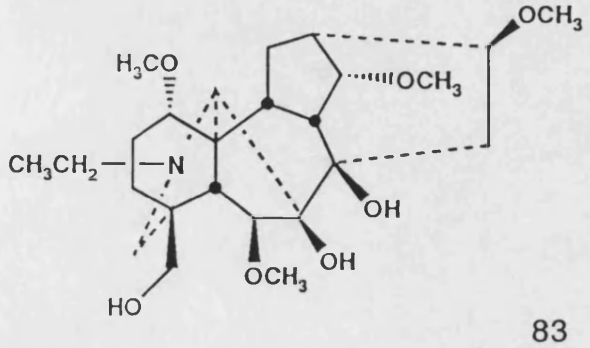


Figure (15) COSY Spectrum of Lycoctonine (2) in $CDCl_3$

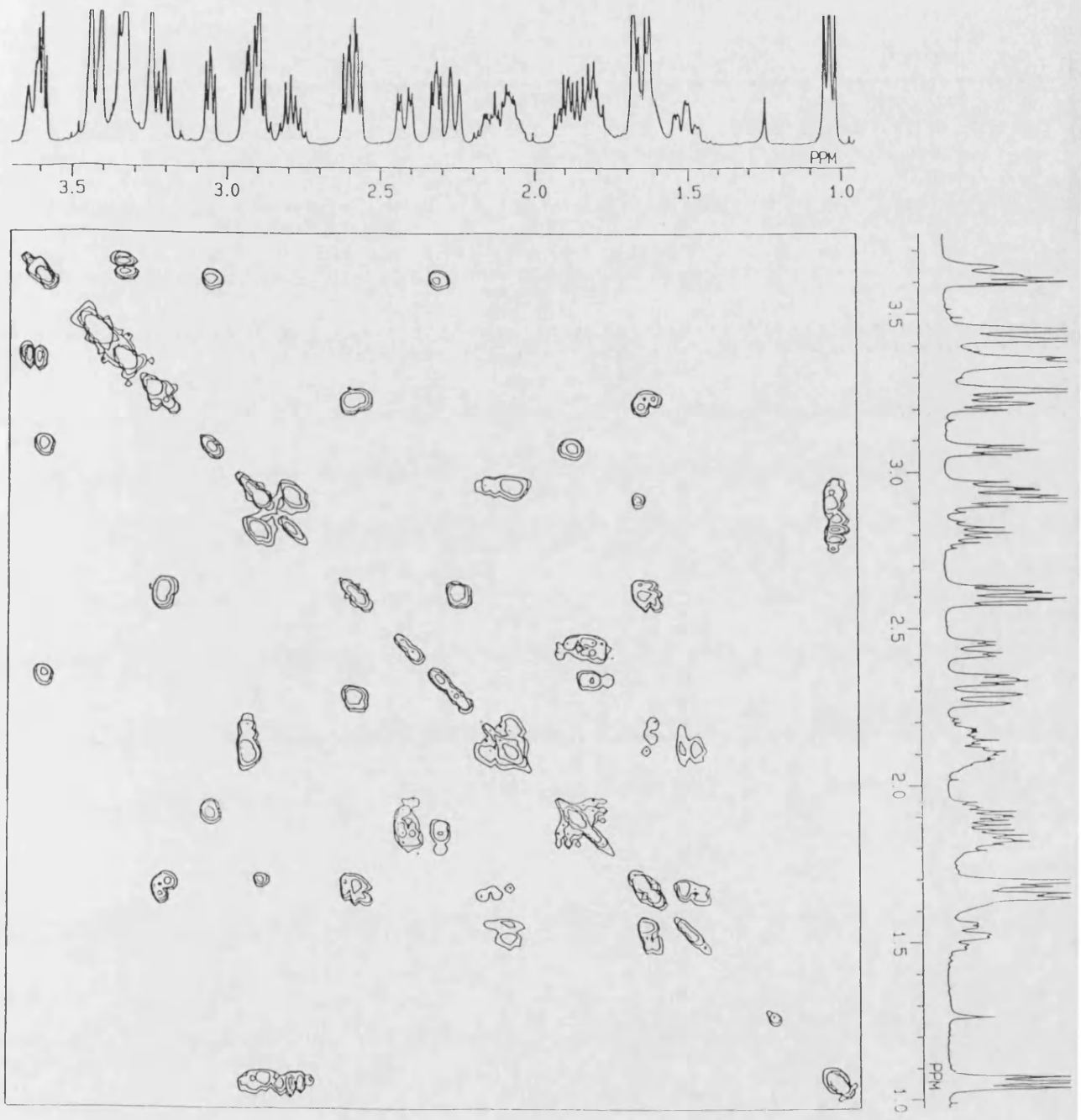


Figure (16) NOESY Spectrum of Lycoctonine (2) in $CDCl_3$

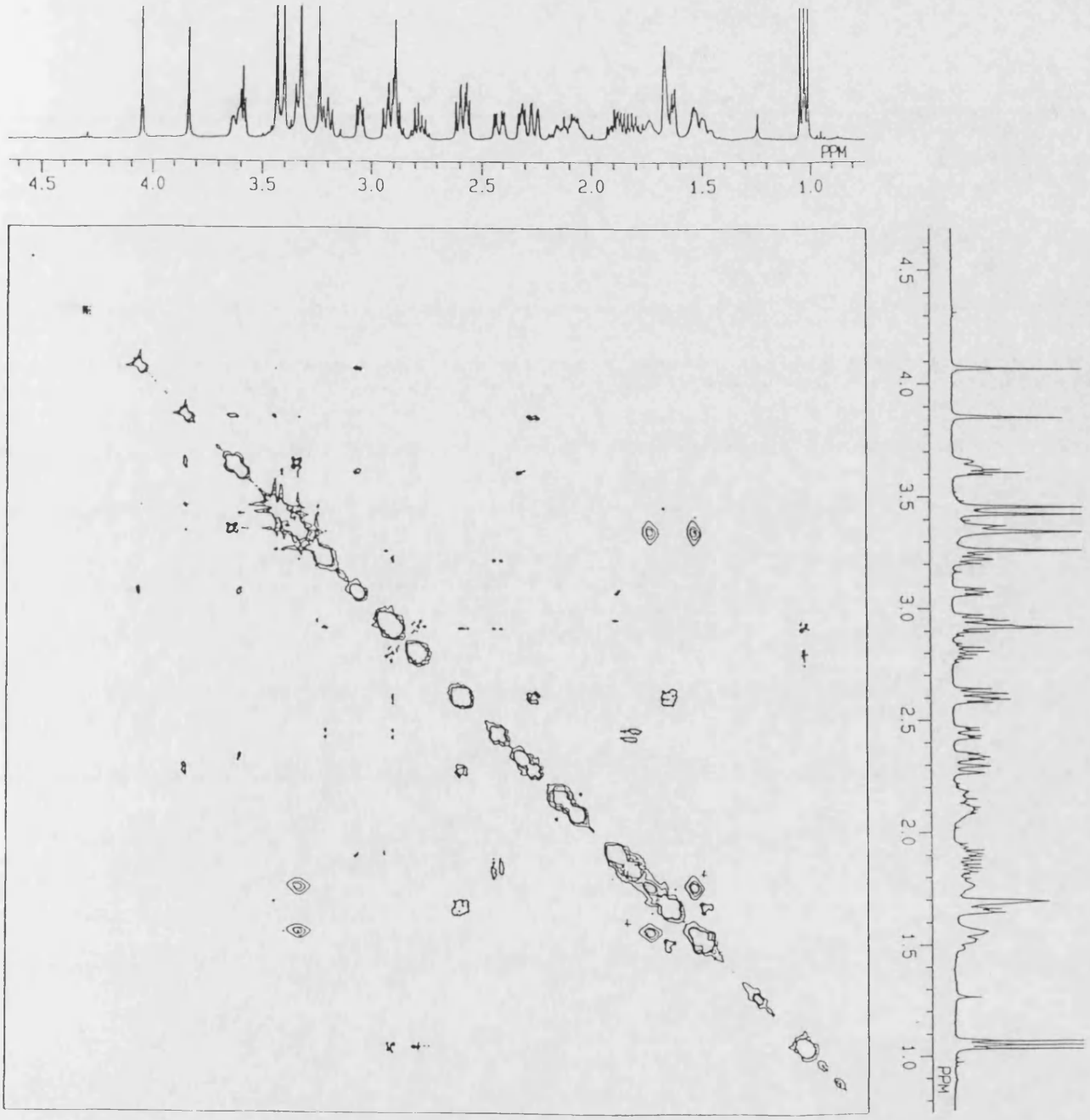


Table 3
NMR Spectral Analysis for Lycoctonine (2)

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
1	84.2	2.96-2.88 (m, H-β-1)	2.20-2.01 (m, H-β-2 and H-α-2) 1.69-1.63 (m, H-9)			3.25 [s, C(1)OC*H*$_3$]
2	26.1	2.20-2.01 (m, H-β-2)	2.96-2.88 (m, H-β-1) 2.20-2.01 (m, H-α-2) 1.59-1.47 (m, H-β-3) 1.69-1.63 (m, H-α-3)			
		2.20-2.01 (m, H-α-2)	2.96-2.88 (m, H-β-1) 2.20-2.01 (m, H-β-2) 1.59-1.47 (m, H-β-3) 1.69-1.63 (m, H-α-3)			
3	31.6	1.59-1.47 (m, H-β-3)	2.20-2.01 (m, H-β-2) 2.20-2.01 (m, H-α-2) 1.69-1.63 (m, H-α-3)		2.27 (dd, H-β-19)	
		1.69-1.63 (m, H-α-3)	2.20-2.01 (m, H-β-2) 2.20-2.01 (m, H-α-2) 1.59-1.47 (m, H-β-3)		2.96-2.88 (m, H-β-1) 3.84 (s, H-6) 2.63-2.57 (m, H-α-19) 3.25 [s, C(1)OC*H*$_3$]	
4	38.5	-				
5	46.1	1.94-1.78 (m, H-5)			2.33 (dd, H-10) 2.96-2.88 (m, H-17) 2.27 (dd, H-β-19)	
6	90.6	3.84 (s, H-6)			1.69-1.63 (m, H-α-3) 1.69-1.63 (m, H-9) 1.75 [br s, C(7)OH]	3.44 [s, C(6)OC*H*$_3$] 3.68-3.59 (m, H-α-18)
7	88.4	-				
8	77.4	-				
9	49.5	1.69-1.63 (m, H-9)	2.33 (dd, H-10) 2.96-2.88 (m, H-β-1)		4.08 [s, C(8)OH]	3.44 [s, C(6)OC*H*$_3$] 2.27 (dd, H-β-19)

Table 3 cont.

Carbon	δ (ppm)	Correlated Protons					
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [NOESY]
10	38.0	2.33 (dd, *J* 4.7, 4.7 and 6.8, H-10)	1.69-1.63 (m, H-9) 1.94-1.78 (m, H-β-12)	$J_{10,12\alpha}$ 4.7 $J_{10,14}$ 4.7 $J_{10,12\beta}$ 6.8			
11	48.8	-					
12	28.7	1.94-1.78 (m, H-β-12)	2.33 (dd, H-10) 2.43 (dd, H-α-12) 3.08-3.05 (m, H-13)				2.96-2.88 (m, H-17)
		2.43 (dd, *J* 14.1, 4.7 and 4.7, H-α-12)	1.94-1.78 (m, H-β-12)	$J_{12\alpha12\beta}$ 14.1 $J_{12\alpha10}$ 4.7 $J_{12\alpha13}$ 4.7	3.21 (dd, H-16)		2.96-2.88 (m, H-17)
13	43.2	3.08-3.05 (m, H-13)	1.94-1.78 (m, H-β-12) 3.68-3.59 (m, H-14)		1.69-1.63 (m, H-β-15)		
14	83.9	3.68-3.59 (m, H-14)	3.08-3.05 (m, H-13)		2.33 (dd, H-10) 3.34 [s, C(16)OC*H*$_3$]		3.41 [s, C(14)OC*H*$_3$] 3.34 [s, C(16)OC*H*$_3$]
15	33.5	1.69-1.63 (m, H-β-15)	2.63-2.57 (m, H-α-15) 3.21 (dd, H-16)		3.08-3.05 (m, H-13) 4.08 [s, C(8)OH]		
		2.63-2.57 (m, H-α-15)	1.69-1.63 (m, H-β-15) 3.21 (dd, H-16)				
16	82.5	3.21 (dd, *J* 8.8 and 7.8, H-16)	1.69-1.63 (m, H-β-15) 2.63-2.57 (m, H-α-15)	$J_{16,15\alpha}$ 8.8 $J_{16,15\beta}$ 7.8	2.43 (dd, H-α-12)		2.96-2.88 (m, H-17) 3.34 [s, C(16)OC*H*$_3$]
17	64.8	2.96-2.88 (m, H-17)			1.94-1.78 (m, H-5) 2.27 (dd, H-β-19) 1.75 [br s, C(7)OH]		1.94-1.78 (m, H-β-12) 2.43 (dd, H-α-12) 3.21 (dd, H-16) 2.63-2.57 (m, H-α-19)
18	67.7	3.35 (d, *J* 9.4, H-β-18)	3.68-3.59 (m, H-α-18)	$J_{18\beta18\alpha}$ 9.4			1.59-1.47 [m, C(18)OH]
		3.68-3.59 (m, H-α-18)	3.35 (d, H-β-18)				3.84 (s, H-6)
19	52.5	2.27 (dd, *J* 11.5 and 1.6, H-β-19)	2.63-2.57 (m, H-α-19)	$J_{19\beta19\alpha}$ 11.5	1.59-1.47 (m, H-β-3) 1.94-1.78 (m, H-5) 2.96-2.88 (m, H-17)	$J_{19\beta3\beta}$ 1.6	
		2.63-2.57 (m, H-α-19)	2.27 (dd, H-β-19)		1.69-1.63 (m, H-α-3)		2.96-2.88 (m, H-17)

Table 3 cont.

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
NCH_2CH_3	51.1	2.87-2.75 (m, 1 of NCH_2 CH_3)	2.96-2.88 (m, 1 of NCH_2 CH_3) 1.04 (t, NCH_2CH_3)			
		2.96-2.88 (m, 1 of NCH_2 CH_3)	2.87-2.75 (m, 1 of NCH_2 CH_3) 1.04 (t, NCH_2CH_3)			
NCH_2CH_3	14.1	1.04 (t, J 7.1, NCH_2CH_3)	2.87-2.75 (m, 1 of NCH_2 CH_3) 2.96-2.88 (m, 1 of NCH_2 CH_3)	$J_{NCH_2CH_3}$ 7.1		
$C(1)OCH_3$	55.8	3.25 [s, $C(1)OCH_3$]			1.69-1.63 (m, H-α-3)	2.96-2.88 (m, H-β-1)
$C(6)OCH_3$	57.9	3.44 [s, $C(6)OCH_3$]				3.84 (s, H-6) 1.69-1.63 (m, H-9)
	-	1.75 [br s, C(7)OH]			3.84 (s, H-6) 2.96-2.88 (m, H-17) 4.08 [s, C(8)OH]	
	-	4.08 [s, C(8)OH]			1.69-1.63 (m, H-9 and H-β-15) 1.75 [br s, C(7)OH]	
$C(14)OCH_3$	57.8	3.41 [s, $C(14)OCH_3$]				3.68-3.59 (m, H-14)
$C(16)OCH_3$	56.2	3.34 [s, $C(16)OCH_3$]			3.68-3.59 (m, H-14)	3.21 (dd, H-16)
	-	1.59-1.47 [m, C(18)OH]				3.35 (d, H-β-18)

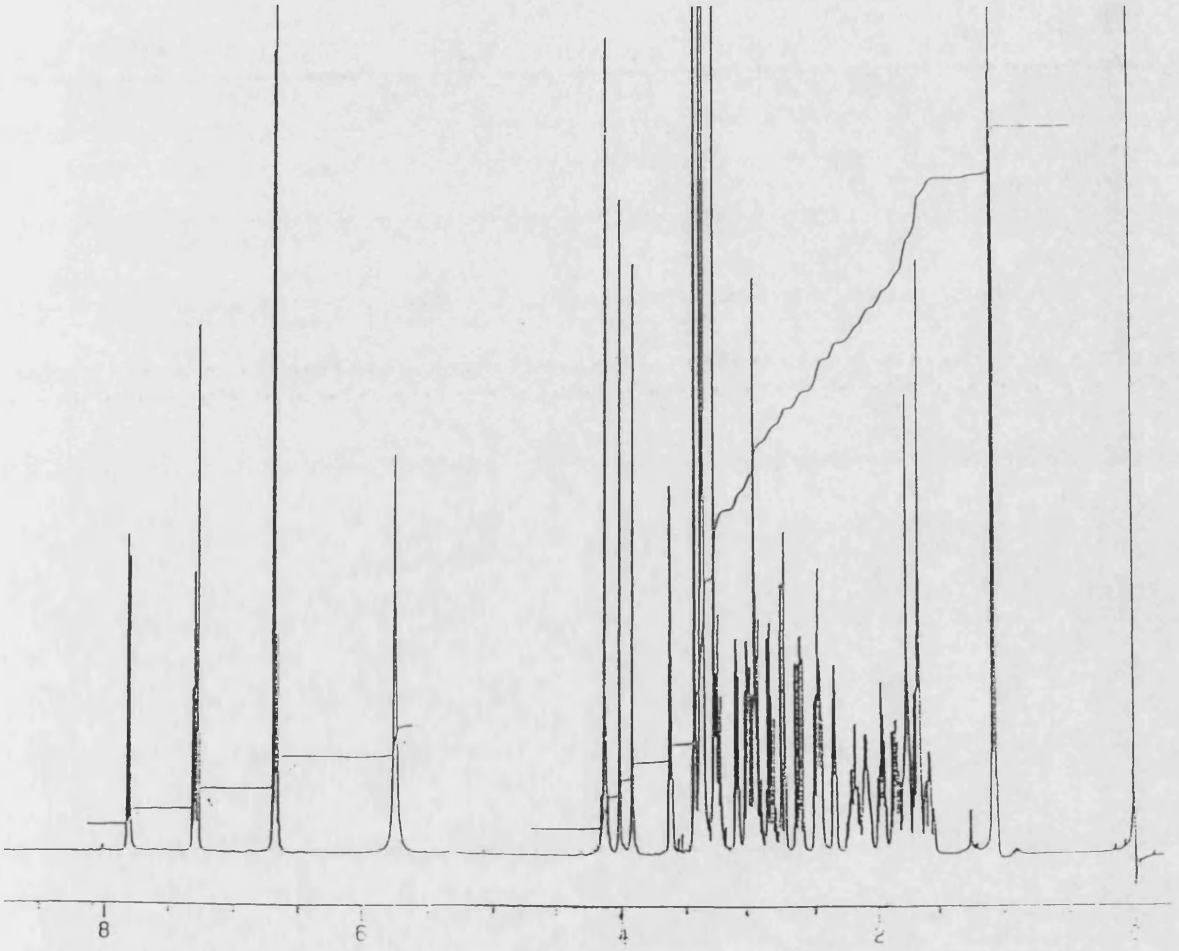
Before D_2O shake, the signals from the CH_2OH hydrogens (H_2-18) are seen as a multiplet (due to coupling between CH-OH hydrogens) with some AB character with a shift difference of A (at δ3.68-3.59ppm) and B (at δ3.35ppm) of δ0.28ppm [Hanuman and Katz (1994) predicted δ0.22±0.07ppm]. H-β-18 and H-α-18 can be seen to interact with a coupling constant of $J_{18\beta 18\alpha} = 9.4$Hz. After a D_2O shake they appear as an AB quartet δ4.00ppm and δ4.24ppm J = approximately 12Hz, because this is a prochiral centre. Assignment of the hydroxyl protons was possible by careful examination of the integration before and after shaking with D_2O. The C(8)-β-OH signal (at δ4.08ppm) was located by its interaction with H-β-15 and H-β-9 and C(7)-β-OH (at δ1.75ppm) was found to interact with both H-6 and H-17 and C(18)OH (at δ1.59-1.47ppm) with H-β-19 and H-β-18 (COSY and long-range COSY spectra).

2.2.7 NMR Assignment of Inuline

The semi-synthesis of the natural product, inuline (40) allowed for the complete and unambiguous characterization by spectroscopic means (Pelletier *et al.*, 1977, Pelletier *et al.*, 1984) (See later in Section 2.2.11 and Section 2.3.2.14). The ^{1}H and ^{13}C NMR signals for the skeletal atoms for inuline were assigned by an analogous approach to that used and discussed for delpheline (219) and MLA (1). Figures (17), (18) and (19) show the ^{1}H NMR spectrum (400MHz) and its expansion and ^{13}C NMR (67.8MHz) spectra and the information obtianed from these together with from the COSY, long-range COSYand NOESY, HETCOR and COLOC spectra has been collated in **Table 4.** In additon, Section 2.2.8 also gives further details of comparison of the values obtained in this study with the literature reviews.

The ^{1}H NMR shifts for the four aromatic protons of the anthranoyl ester group have been unambiguously assigned. A pattern of these protons consisting of a doublet (dd), a triplet (dt/ddd), and a multiplet is observed [Figure (17)]. Expansion of the ^{1}H NMR spectrum [Figure (18)] revealed that the doublet of doublets at δ7.79ppm for H-6' has coupling constants $J_{6'5'}$ = 8.4Hz and $J_{6'4'}$ = 1.6Hz, and the "triplet" at δ7.27ppm for H-4' has $J_{4'3'}$ = 8.1Hz or 7.4Hz, $J_{4'5'}$ = 7.4Hz or 8.1Hz (these two *ortho* coupling constants are indistinguishable from each other) and $J_{4'6'}$ = 1.6Hz as before. From the two proton multiplet upfield in the aromatic region for H-3' and H-5', as well as the previously mentioned relevant coupling constants ($J_{3'4'}$, $J_{5'4'}$ and $J_{5'6'}$), it was possible to pick out the other *meta* coupling constant $J_{5'3'}$ = $J_{3'5'}$ = 2.1Hz and the *para* one $J_{3'6'}$ = 0.9Hz ($J_{6'3'}$ was not observed in the signal for H-6').

Figure (17) ^1H NMR (400MHz) Spectrum of Inuline (40) in $CDCl_3$



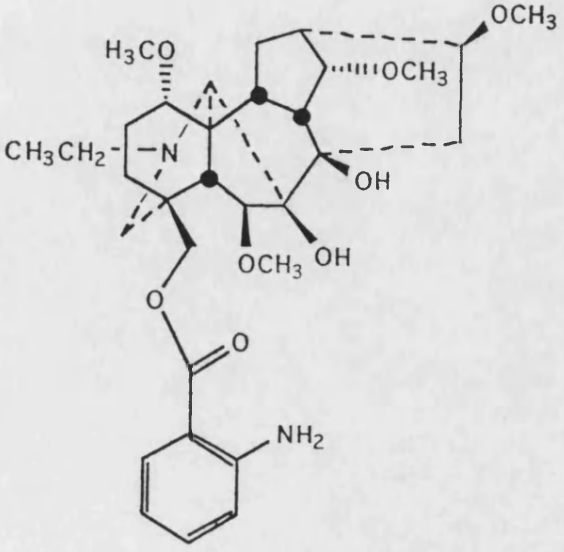


Figure (18) Expansion of ^1H NMR (400MHz) Spectrum of Inuline (40) in $CDCl_3$

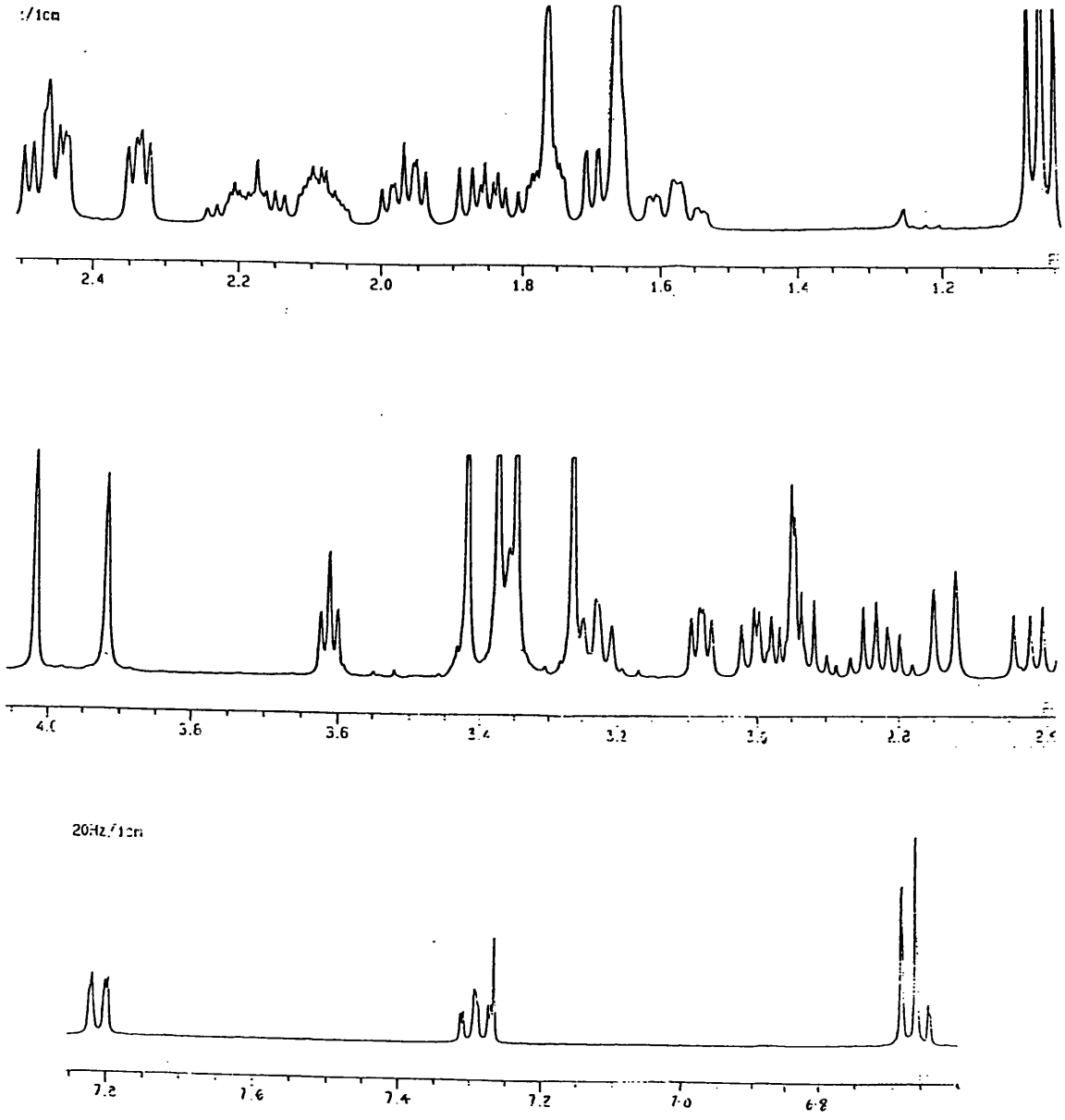
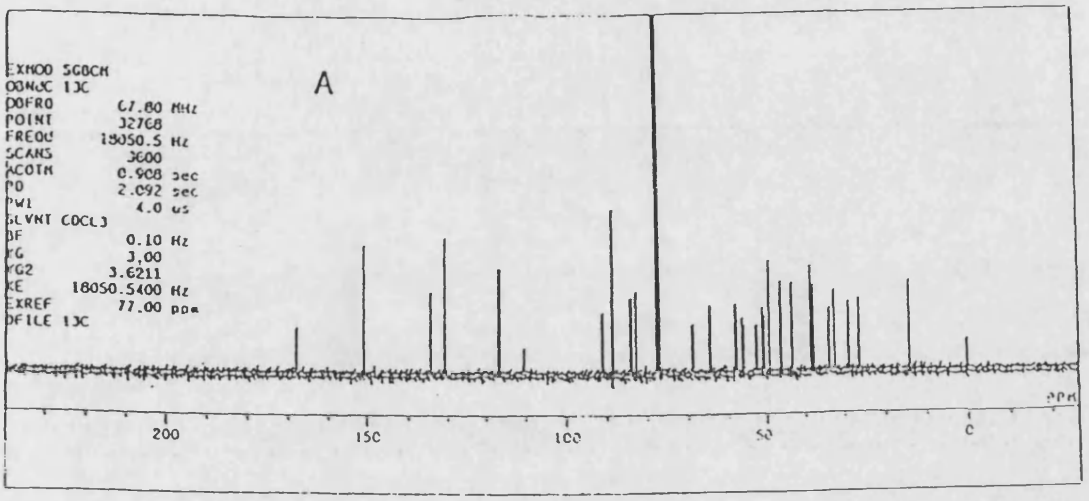


Figure (19) ^{13}C NMR (67.8MHz) Spectra of Inuline (40) in $CDCl_3$:
Full Spectrum **(A)** and DEPT 135° Subspectrum showing Methine and Methyl Carbons **Up** and Methylene Carbons **Down (B)**



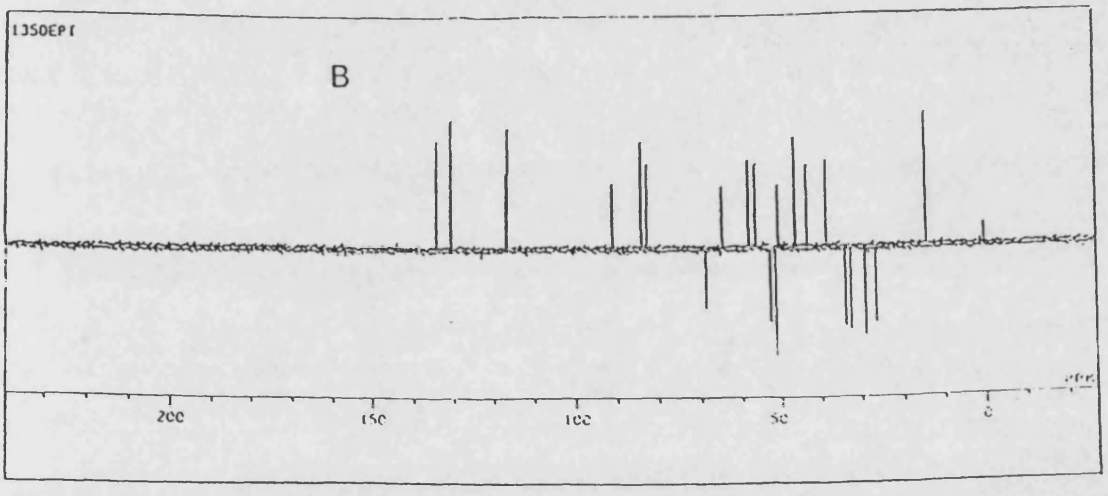


Table 4
NMR Spectral Analysis for Inuline (40)

Carbon	δ (ppm)	Correlated Protons					
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [NOESY]
1	84.1	3.00 (dd, *J* 7.0 and 10.1, H-β-1)	2.25-2.03 (m, H-β-2)	$J_{1\beta2\beta}$ 10.1	1.63-1.52 (m, H- β-3)	$J_{1\beta3\beta}$ 7.0	3.27 [s, C(1)OC*H*$_3$]
2	26.2	2.25-2.03 (m, H-β-2)	3.00 (dd, H-β-1) 2.25-2.03 (m, H-α-2) 1.63-1.52 (m, H-β-3)				
		2.25-2.03 (m, H-α-2)	2.25-2.03 (m, H-β-2) 1.79-1.74 (m, H-α-3)				
3	32.3	1.63-1.52 (m, H-β-3)	2.25-2.03 (m, H-β-2) 1.79-1.74 (m, H-α-3)		3.00 (dd, H-β-1)		2.50-2.42 (m, H-β-19)
		1.79-1.74 (m, H-α-3)	2.25-2.03 (m, H-α-2) 1.63-1.52 (m, H-β-3)				
4	37.7	-					
5	50.4	1.72-1.65 (m, H-5)			2.96-2.89 (m, H-17) 2.50-2.42 (m, H-β-19)		2.00-1.93 (m, H-10)
6	91.0	3.93 (s, H-6)					3.41 [s, C(6)OC*H*$_3$]
7	88.6	-					
8	77.6	-					
9	43.3	3.08 (dd, *J* 4.6 and 6.7, H-9)	2.00-1.93 (m, H-10) 3.61 (dd, H-14)	$J_{9,14}$ 4.6 $J_{9,10}$ 6.7			1.79-1.74 [m, C(8)OH]

Table 4 cont.

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
10	46.2	2.00-1.93 (m, H-10)	3.08 (dd, H-9) 1.89-1.80 (m, H-β-12)		3.61 (dd, H-14)	1.72-1.65 (m, H-5)
11	49.1	-				
12	28.8	1.89-1.80 (m, H-β-12)	2.00-1.93 (m, H-10) 2.50-2.42 (m, H-α-12) 2.34 (dd, H-13)			
		2.50-2.42 (m, H-α-12)	1.89-1.80 (m, H-β-12)		3.24-3.19 (m, H-16)	
13	38.3	2.34 (dd, *J* 4.6 and 7.0, H-13)	1.89-1.80 (m, H-β-12) 3.61 (dd, H-14)	$J_{13,14}$ 4.6 $J_{13,12\beta}$ 7.0	1.72-1.65 (m, H-β-15)	
14	84.0	3.61 (dd, *J* 4.6 and 4.6, H-14)	3.08 (dd, H-9) 2.34 (dd, H-13)	$J_{14,9}$ 4.6 $J_{14,13}$ 4.6	2.00-1.93 (m, H-10)	3.35 [s, C(14)OC*H*$_3$]
15	33.7	1.72-1.65 (m, H-β-15)	2.61 (dd, H-α-15)		2.34 (dd, H-13) 1.79-1.74 [m, C(8)OH]	
		2.61 (dd, *J* 8.9 and 15.3, H-α-15)	1.72-1.65 (m, H-β-15) 3.24-3.19 (m, H-16)	$J_{15\alpha16}$ 8.9 $J_{15\alpha15\beta}$ 15.3		
16	82.7	3.24-3.19 (m, H-16)	2.61 (dd, H-α-15)		2.50-2.42 (m, H-α-12)	3.38 [s, C(16)OC*H*$_3$]
17	64.6	2.96-2.89 (m, H-17)			1.72-1.65 (m, H-5) 2.50-2.42 (m, H-β-19)	1.72-1.65 [m, C(7)OH]
18	68.7	4.18-4.01 (m, H-β-18)	4.18-4.01 (m, H-α-18)			
		4.18-4.01 (m, H-α-18)	4.18-4.01 (m, H-β-18)			
19	52.6	2.50-2.42 (m, H-β-19)	2.75-2.72 (m, H-α-19)		1.72-1.65 (m, H-5) 2.96-2.89 (m, H-17)	1.63-1.52 (m, H-β-3)
		2.73 (d, *J* 11.6, H-α-19)	2.50-2.42 (m, H-β-19)	$J_{19\alpha19\beta}$ 11.6		

Table 4 cont.

Carbon	δ (ppm)	Correlated Protons				
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	^{1}H-^{1}H [NOESY]
NCH_2CH_3	51.1	2.87-2.77 (m, 1 of NCH_2 CH_3)	2.96-2.89 (m, 1 of NCH_2 CH_3) 1.07 (t, NCH_2CH_3)			
		2.96-2.89 (m, 1 of NCH_2CH_3)	2.87-2.77 (m, 1 of NCH_2 CH_3) 1.07 (t, NCH_2CH_3)			
NCH_2CH_3	14.1	1.07 (t, *J* 7.0, NCH_2CH_3)	2.87-2.77 (m, 1 of NCH_2 CH_3) 2.96-2.89 (m, 1 of NCH_2 CH_3)	$J_{NCH_2CH_3}$ 7.0		
$C(1)OCH_3$	55.9	3.27 [s, $C(1)OCH_3$]				3.00 (dd, H-β-1)
$C(6)OCH_3$	58.0	3.41 [s, $C(6)OCH_3$]				3.93 (s, H-6)
	-	1.72-1.65 [m, C(7)OH]			1.79-1.74 [m, C(8)OH]	2.96-2.89 (m, H-17)
	-	1.79-1.74 [m, C(8)OH]				3.08 (dd, H-9) 1.72-1.65 [m, H-β-15 and C(7)OH]
$C(14)OCH_3$	57.9		3.61 (dd, H-14)			3.35 [s, $C(14)OCH_3$]
$C(16)OCH_3$	56.3		3.24-3.19 (m, H-16)			3.38 [s, $C(16)OCH_3$]

Table 4 cont.

Carbon	δ (ppm)	Correlated Protons					
		^{13}C-^{1}H (one bond)	^{1}H-^{1}H [COSY] (two and three bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [Long-Range COSY] (four and more bond)	Proton Coupling Constant (Hz)	^{1}H-^{1}H [NOESY]
C=O	167.9	-					
1'	110.4	-					
2'	150.9	-					
3'	117.0	6.70-6.64 (m, H-3')	7.27 (ddd, H-4')		6.70-6.64 (m, H-5')		
4'	134.4	7.27 (ddd, *J* 8.1, 7.4 and 1.6, H-4')	6.70-6.64 (m, H-3' and H-5')	$J_{4'3'}$ 8.1 or 7.4 $J_{4'5'}$ 7.4 or 8.1	7.79 (dd, H-6')	$J_{4'6'}$ 1.6	
5'	116.3	6.70-6.64 (m, H-5')	7.27 (ddd, H-4') 7.79 (dd, H-6')		6.70-6.64 (m, H-3')		
6'	130.8	7.79 (dd, *J* 8.4 and 1.6, H-6')	6.70-6.64 (m, H-5')	$J_{6'5'}$ 8.4	7.27 (ddd, H-4')	$J_{6'4'}$ 1.6	
	-	5.77 (br s, NH_2)					

From the spectra it was possible to pick out, amongst others, the following coupling constants: $J_{3\beta3\alpha}$ = 13.0Hz, $J_{3\beta19\beta}$ (W-coupling) = 3.4Hz, $J_{19\beta17}$ 5.8Hz, $J_{5,10}$ or $J_{5,17}$ = 7.0Hz, $J_{10,9}$ = 6.7Hz, $J_{10,12\beta}$ =7.0Hz, $J_{10,12\beta}$ = 4.9Hz, $J_{12\beta12\alpha}$= 14.3Hz, $J_{12\beta13}$ = 7.0Hz, $J_{16,15\alpha}$ = 8.9Hz. For Inuline (40), it can be seen that a multiplet (at δ2.96-2.89ppm) is observed for H-17, whereas most norditerpenoid alkaloids give a broad singlet at δ3.02±0.22ppm represent this methine (Hanuman and Katz, 1994). The splitting is due to coupling with neighbouring protons, H-β-19 and H-5.

The signals for the AB system of the methylene protons of the *N*-ethyl side-chain display appear as a multiplet at δ2.96-2.89ppm and the terminal methyl group appears as a triplet at δ1.07ppm. H_2-18 also displays AB character but a value for $J_{18\beta18\alpha}$ is difficult to establish.

2.2.8 Comparison of NMR Spectral Analysis for Norditerpenoid Alkaloids

In **Table 5** ^{13}C and ^{1}H NMR spectral data has been compiled from our laboratory and from the reviews of Hanuman and Katz (1994) and Pelletier *et al.* (1984). By averaging over a large number of compounds having similar structural features, the general ranges in chemical shifts for the various skeletal and functional groups make excellent templates for the confident assignment of other related C_{19}-diterpenoid alkaloids, with subtly different substitution patterns and stereochemistry around the complex skeleton. A comprehensive catalogue of spectral data for a group of compounds as structurally complex as these alkaloids, with so many different permutations of oxygenation around the skeleton, would be of great importance. One can quickly pick out diagnostic peaks and regions in a spectrum and by comparing and contrasting in this way, the chemical shifts (and multiplicities where relevant) for the different functional groups can be predicted, depending on the neighbouring groups. For example, the slightly ambiguous assignments of the protons for the methines at C-3 and C-15 plus C-10 and C-13 for MLA, delpheline, lycoctonine and inuline in our studies were facilitated and backed up by such information and it is easy to see if a signal or assignment has deviated or to establish why an apparent trend has been strayed from.

When considering only, the MLA, inuline and lycoctonine series, the only assignments which are notable different for the skeletal carbon atoms are the three methines of ring C, C-9, C-10, and C-13 for lycoctonine and in the ^{1}H NMR spectra, the most deviant protons chemical shift assignments include as well as, H-9, H-10, and H-13, those for hydrogens near the C(18) oxygenated function, namely, H-α-3, H-5, H-α-18, H-β-18, and H-α-19.

Table 5
Comparison of NMR Spectral Analysis for Norditerpenoid Alkaloids

Position	According to Pelletier *et al.*		According to Hanuman and Katz	According to Hanuman and Katz	According to This Study	
	Carbon δ/(ppm)	Proton δ/(ppm)	Tabulated Proton δ/(ppm)	Text Proton δ/(ppm)	Carbon δ/(ppm)	Proton δ/(ppm)
1	80.0-77.0 [with C(1)OCH_3 and C(18)H_3]		2.94±0.01 (for H-β-1)	3.04±0.11 (for H-β-1)	82.7	3.02 (for H-β-1)
	85.5-83.0 [with C(1)OCH_3, general range]				84.1-83.9	2.95-2.92, 2.96-2.88, 3.00 (for H-β-1)
2	27.0-25.5 [with C(1)OCH_3 and without C(3)OR]		2.00±0.10 [for H-β-2 with C(1)OCH_3]	2.10±0.20 [for H-β-2 with C(1)OCH_3]	26.7-26.1	2.07-1.98, 2.15-2.03, 2.20-2.01, 2.25-2.03 (for H-β-2)
			2.08±0.08 [for H-α-2 with C(1)OCH_3]	2.10±0.20 [for H-α-2 with C(1)OCH_3]		2.21-2.08, 2.20-2.15, 2.20-2.01, 2.25-2.03 (for H-α-2)
3	39.5-36.0 [with C(1)OCH_3 and C(18)H_3]		1.39±0.12 [for H-β-3 with C(1)OCH_3]	1.59±0.20 [for H-β-3 with C(1)OCH_3]	36.9	1.26-1.16 (for H-β-3)
			1.67±0.16 [for H-α-3 with C(1)OCH_3]	1.59±0.20 [for H-α-3 with C(1)OCH_3]		1.59 (for H-α-3)
			1.39±0.12 [for H-β-3 with C(1)OCH_3]	1.59±0.20 [for H-β-3 with C(1)OCH_3]	32.3-31.6	1.63-1.47 (for H-β-3)
			1.67±0.16 [for H-α-3 with C(1)OCH_3]	1.59±0.20 [for H-α-3 with C(1)OCH_3]		1.79-1.63 (for H-α-3)
4	35.0-32.0 [with C(18)H_3 and C(3)H_2]	-	-	-	33.8	-
	41.0-37.0 [with C(18)H_2OR and C(3)H_2]	-	-	-	38.5-37.6	-
5	52.5-50.5 [with C(1)OCH_3 and C(6)OH]		1.59±0.11 [for H-5 with C(6)OH]		56.6	1.22
	46.5-42.5 [with C(6)OCH_3]		1.78±0.09 [for H-5 with C(6)OCH_3]		50.3, 46.1, 50.4	1.70-1.64, 1.94-1.78, 1.72-1.65
6	79.5-77.0 [with C(6)OH and C(7)OCH_2OC(8)]		4.41±0.09 [for H-α-6 with C(6)OH]		79.2	4.19 (for H-α-6)
	92.0-89.5 [with C(6)OCH_3]	4.20-3.90 [for H-α-6 with C(6)OCH_3]	3.92±0.07 [for H-α-6 with C(6)OCH_3]		91.0-90.6	3.85, 3.84, 3.93 (for H-α-6)
7	93.5-90.5 [with C(7)OCH_2O]	-	-	-	92.7	-
	89.0-87.5 [with C(7)OH]	-	-	-	88.5, 88.4, 88.6	-
8	84.0-81.5 [with C(8)OCH_2O and without C(14)ketone]	-	-	-	84.1	-
	78.5-76.0 [with C(8)OH and C(7)OH]	-	-	-	77.6-77.4	-
9			3.10±0.14 [for H-9 with C(8)OH and C(14)OCH_3]		40.3 43.2, 49.5, 43.3	3.67-3.60 3.10-3.03, 1.69-1.63, 3.08

Table 5 cont.

Position	According to Pelletier *et al.*		According to Hanuman and Katz	According to Hanuman and Katz	According to This Study	
	Carbon δ/(ppm)	Proton δ/(ppm)	Tabulated Proton δ/(ppm)	Text Proton δ/(ppm)	Carbon δ/(ppm)	Proton δ/(ppm)
10	43.0-37.5 [without C(9)OH]		1.94±0.04 [for H-10 with C(8)OH and C(14)OCH_3]	1.84±0.21 [for H-10 with C(8)OH and C(14)OCH_3]	47.7, 46.1, 38.0, 46.2	2.21-2.08, delpheline 1.98-1.90, 2.33, 2.00-1.93
11	51.5-47.0 (general)	-	-	-	50.4-48.8	-
12	32.0-26.3 [with C(10) and C(13) unsubstituted]		1.75±0.04 [for H-β-12 with C(8)OH and C(14)OCH_3]	1.69±0.28 [for H-β-12 with C(8)OH and C(14)OCH_3]	28.8-28.1	1.86-1.78 (for H-β-12), delpheline 1.90-1.78 (for H-β-12)
			2.25±0.17 [for H-α-12 with C(8)OH and C(14)OCH_3]	2.26±0.29 [for H-α-12 with C(8)OH and C(14)OCH_3]		2.55 (for H-α-12), delpheline 2.50-2.42 (for H-α-12)
13	46.5-43.0 (general)		2.40±0.07 [for H-13 with C(8)OH and C(14)OCH_3]		37.7, 38.0, 43.2, 38.3	2.37, delpheline 2.35-2.31, 3.08-3.05, 2.34
14	77.0-74.5 [with C(14)OR and C(9), C(10), and C(13) unsubstituted]	4.10, 3.85-3.60 varies (for H-β-14)	3.65±0.05 [for H-β-14 with C(8)OH and C(14)OCH_3]	3.65±0.05 [for H-β-14 with C(8)OH and C(14)OCH_3]	84.0-83.0	3.71-3.64 (for H-β-14), delpheline 3.68-3.59 (for H-β-14)
15	36.9-32.5 (lycoctonine type)		1.69±0.09 [for H-β-15 with C(8)OH and C(14)OCH_3]	1.69±0.09 [for H-β-15 with C(8)OH and C(14)OCH_3]	33.7-33.4	1.86-1.78 (for H-β-15), delpheline 1.72-1.63 (for H-β-15)
			2.80±0.20 [for H-α-15 with C(8)OH and C(14)OCH_3]	2.80±0.20 [for H-α-15 with C(8)OH and C(14)OCH_3]		2.49 (for H-α-15), delpheline 2.64-2.56 (for H-α-15)
16	84.5-79.5 [with C(16)OCH_3]		3.29±0.10 [for H-α-16 with C(8)OH and C(14)OCH_3]	3.29±0.10 [for H-α-16 with C(8)OH and C(14)OCH_3]	82.7-81.8	3.25-3.19 (for H-α-16), delpheline 3.24-3.17 (for H-α-16)
17	66.5-63.5 (lycoctonine type)				64.8-63.6	3.08, 2.95-2.92, 2.96-2.88, 2.96-2.89
C(18)H_3	26.0-24.5 (lycoctonine type)	1.10-0.8			25.3	0.93
C(18)H_2OCOR	70.5-68.5 (lycoctonine type)			0.22±0.07 (for H-β-18)	69.5, 68.7, MLA, Inuline	4.15-4.00, 4.18-4.01 (for H-β-18), MLA, Inuline
				0.22±0.07 (for H-α-18)		4.15-4.00, 4.18-4.01 (for H-α-18), MLA, Inuline
C(18)H_2OH	68.5-66.5 (general)			0.22±0.07 (for H-β-18)	67.7	3.35 (for H-β-18)
				0.22±0.07 (for H-α-18)		3.68-3.59 (for H-α-18)
19	57.5-52.5 (general)			2.14±0.16 (for H-β-19)	57.4, 52.5	2.27-2.24 (for H-β-19)
				2.56±0.22 (for H-α-19)		2.69-2.57 (for H-α-19)
	52.5-52.0 [with C(18)H_2OCOR]			2.14±0.16 (for H-β-19)	52.4, 52.6, MLA, Inuline	2.50-2.38 (for H-β-19)
				2.56±0.22 (for H-α-19)		2.73-2.69 (for H-α-19)

Table 5 cont.

Position	According to Pelletier *et al.*		According to Hanuman and Katz	According to Hanuman and Katz	According to This Study	
	Carbon δ/(ppm)	Proton δ/(ppm)	Tabulated Proton δ/(ppm)	Text Proton δ/(ppm)	Carbon δ/(ppm)	Proton δ/(ppm)
NCH_2 CH_3	48.0-46.0 (general)			2.89±0.05 (for 1 of NCH_2 CH_3)	50.6, 50.9, 51.1, 51.1	2.69-2.60, 2.73-2.69, 2.87-2.75, 2.87-2.77 (for 1 of NCH_2 CH_3)
				2.89±0.05 (for 1 of NCH_2 CH_3)		2.77, 2.95-2.92, 2.96-2.88, 2.96-2.89 (for 1 of NCH_2 CH_3)
NCH_2CH_3	14.5-13.0 (general)	1.15-0.95		1.08±0.05	14.1-13.8	1.07-1.04
C(1)OCH_3	57.0-55.5 (general)	3.90-3.10			55.9-55.5	3.28-3.25
C(6)OH	-				-	3.34
C(6)OCH_3	58.0-56.5 (general)	3.90-3.10			58.1-57.9	3.45-3.41
C(7)OCH_2 OC(8)	94.5-92.5 [for OCH_2O without C(6)ketone]	5.55-4.80 (for OCHα O)			92.9	5.05 (for OCHα O)
		5.55-4.80 (for OCHβ O)				5.13 (for OCHβ O)
C(7)OH	-				-	1.75, 1.72-1.65
C(8)OH	-				-	4.08, 1.79-1.74
C(14)OCH_3	58.5-57.5 (general)	3.90-3.10			57.9-57.8	3.43, 3.42, 3.41, 3.35
C(16)OCH_3	58.5-55.5 (general)	3.90-3.10			56.3-56.2	3.38-3.34
C(18)OH	-				-	1.59-1.47

Table 5 cont.

Position	According to Pelletier *et al.*		According to Hanuman and Katz	According to Hanuman and Katz	According to This Study	
	Carbon δ/(ppm)	Proton δ/(ppm)	Tabulated Proton δ/(ppm)	Text Proton δ/(ppm)	Carbon δ/(ppm)	Proton δ/(ppm)
C=O		-			164.1, 167.9, MLA, Inuline	-
1'		-			126.9, 110.4, MLA, Inuline	-
2'		-			133.1, 150.9, MLA, Inuline	-
3'		8.75-6.40			130.0, 117.0, MLA, Inuline	7.30, 6.70-6.64, MLA, Inuline
4'		8.75-6.40			133.7, 134.4, MLA, Inuline	7.70, 7.27, MLA, Inuline
5'		8.75-6.40			129.4, 116.3, MLA, Inuline	7.55, 6.70-6.64, MLA, Inuline
6'		8.75-6.40			131.0, 130.8, MLA, Inuline	8.05, 7.79, MLA, Inuline
NH_2	-				-	5.77
1"		-			179.8	-
2"					35.2	3.02-2.96
3"					37.0	2.56-2.50 1 of H_2-3")
						3.10-3.03 (1 of H_2-3")
4"		-			175.8	-
5"					16.4	1.47 (H_3-5")

In the methoxyl region of the ^{13}C NMR spectra, the three alkaloids show consistent chemical shift trend, with C(6)O*C*H_3 more downfield than C(14)O*C*H_3, than C(16)O*C*H_3, than C(1)O*C*H_3. But it appears that in the ^1H NMR spectrum of inuline, that C(14)OCH_3 and C(16)OCH_3 may be transposed (Pelletier *et al.*, 1984, Pelletier *et al.*, 1977, and Jones and Benn, 1973).

The diagnostic region for the methoxy signals is often overlapping with the C-19 signal (which will be split in the HETCOR spectrum to reflect correlation with the two different proton environments for H_2-19, α and β). Also overlapping, in this region of the ^{13}C NMR spectrum, is the C-5 signal. This unusual downfield shift of the C-5 methine is correlated with an upfield signal. Such a chemical shift is comparable with that found in 2-*t*-butylcyclohexan-1-ol of 53.8ppm.

As expected, the chemical shifts for the aromatic protons in the side-chains of MLA and inuline are different, due the different nitrogen substituent at C(2'). However, it has been shown that the protons display the same trend, tending from downfield H-6', to H-4', to H-5', to upfield H-3', in both cases. In the ^{13}C NMR spectra for inuline, the signal for C-2' at 150ppm is considerably higher than the corresponding carbon in MLA, and the trend in chemical shift, in each case, is not comparable with the one observed in the ^1H NMR spectra, this time moving upfield from C-4', to C-6', to C-3', to C-5' to C-1'.

2.2.9 MS Fragmentation Patterns of Norditerpenoid Alkaloids

The mass spectra of diterpenoid alkaloids have been studied by a group of Russian researchers and reviewed, over the period 1985 to 1992, by Yunusov (1991 and 1993). The mode of cleavage of some of the fragments was considered and the measurement of the ratio of metastable and parent ion peak heights made as an indicator. The cleavage of fragments in norditerpenoid alkaloids to give $(M-R)^+$ and $(M-OR)^+$ is commonly observed and the Russian workers propose that the fragments result from cleavage at C-1 or C-6 such that, for alkaloids possessing $C(1)OCH_3$ a high intensity of (M-31) ions, for the ejection of the methoxyl radical at C-1 is expected, and for alkaloids possessing $C(6)OCH_3$, C(7)OH, and C(8)OH a high intensity of (M-15) ions, at the expense of $C(6)OCH_3$, is predicted (Yunusov, 1991). Thus, for alkaloids such as MLA (1), lycoctonine (2), inuline (40), possessing $C(1)OCH_3$, $C(6)OCH_3$, C(7)OH, and C(8)OH, fragmentations corresponding to the loss of both CH_3 and OCH_3 are usually seen.

We observe that for delpheline (219), [(+) and (-) FAB MS, respectively, in *m*-nitrobenzyl alcohol (*m*NBA)] peaks for the pseudo molecular ion [MH^+, m/z 450 and $(M-H)^-$, m/z 448] and for either $(MH-OCH_3)^+/(M-OCH_2)^+$ or $(M-OCH_3)^{\cdot}/(M-H-OCH_2)^{\cdot}$ [m/z 419/m/z 418] was revealed. Ejection of a formaldehyde molecule at the expense of the 7,8-methylenedioxy group is a common fragmentation for such alkaloids (Yunusov, 1991). (M-17) and (M-15) ions were anticipated but were not observed on this occasion, for the ejection of an hydroxyl radical from C-6 and for the loss of CH_3 (from the *N*-ethyl group) (See Section 2.3.2.5).

The mass spectrum for MLA (1) revealed a pseudo molecular ion peak [(+) FAB, MH^+, m/z 683] and a peak at m/z 216 is observed in (+)FAB, as seen in the synthetic analogues in Chapter 4, so suggesting the presence of the same acyl moiety) (See Section 2.3.2.6). Other peaks are seen for the losses of CH_3 and

OCH_3 as expected. Likewise, (+) FAB mass spectroscopy showed a pseudo molecular ion peak for lycoctonine (2) (MH^+, m/z 468) and a peak for $(M\text{-}OCH_3)^+$ (m/z 436), but not $(M\text{-}CH_3)^+$ (See Section 2.3.2.7). For inuline (40), (+) FAB MS fragmentation revealed a pseudo molecular ion peak (MH^+, m/z 587) and $(M\text{-}CH_3)^+$ (m/z 571) (See Section 2.3.2.14). In addition, a peak at m/z 120 for $C_7H_6NO^+$ is observed.

2.2.10 Establishment of the Stereochemistry of the Methyl Succinimide Moiety of MLA

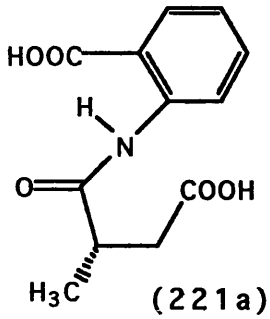
As Benn and his colleagues have consistently drawn (Sun *et al.*, 1991, Sun and Benn, 1992, and Majak *et al.*, 1987), the methylsuccinimido moiety of MLA (1) is apparently derived from *S*-(-)-methylsuccinic acid (Chemical Abstracts Registry Number [2174-58-5]); though, many authors have left the stereochemistry of this methyl substituent as ambiguous. Indeed, as recently as 1989 and 1993, the chirality at this carbon centre has been left undefined and must, therefore, be supposed to be undefined or insecure (Pelletier *et al.*, 1987, Pelletier *et al.*, 1989a and Kraus *et al.*, 1993). Early work by Goodson (1943) showed that *S*-(-)-methylsuccinic acid was one of the hydrolysis products from (1), although the specific optical rotation found was small and no inference was made as to the stereochemistry of the carbon bearing this methyl group. Therefore, we undertook a proof of the configuration of this remaining chiral centre in natural MLA (1) to aid in our modelling of the nicotinic pharmacophore for more accurate interpretation of SAR data.

One method by which this remaining stereocentre could be characterized is by comparison of the optical rotation of methylsuccinic acid obtained from the natural product with that of methylsuccinic acid prepared with known stereochemistry. Another method is by comparison of the ^{13}C NMR spectra of the corresponding *l*-dimenthyl esters.

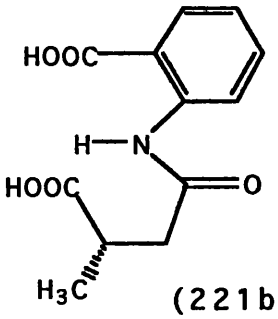
The treatment of MLA with alkali yielded a mixture of *N*-[2-(*S*)-methylsuccinyl]anthranilic acid and *N*-[3-(*S*)-methylsuccinyl]anthranilic acid, (221a) and (221b), as well as lycoctonine (2) (See Section 2.3.2.7). Figure (20) shows the ^1H NMR spectrum (270MHz) of (221a) and (221b). These half-acid amides were used to obtain natural methylsuccinic acid (222), by acid catalyzed hydrolysis (See Section 2.3.2.8). Comparison of the specific rotation with literature values indicated that the methylsuccinic moiety is *S*. Figure (21) (A) shows the ^1H NMR spectrum (270MHz) of natural *S*-(-)-methylsuccinic acid (222).

Synthetic *S*-(-)-methylsuccinic acid (223) was prepared by hydrogenation of itaconic acid (224) in the presence of a $RhCl_3$/(2*S*,4*S*)-1-*tert*-butyl 4-(diphenylphosphino)-2-(diphenylphosphinomethyl)-1-pyrrolidinecarboxylate (225))/(*S*)-1-phenylethylamine optically active catalyst (226) (72% yield, >90% ee based on *R*-enantiomer $[\alpha]_D$ = +15.5°) (See Section 2.3.2.9).

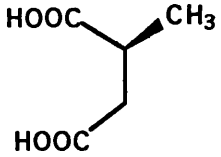
The scheme shows the proposed mechanism, in brief, for this asymmetric transfer-hydrogenation, which avoids the use of gaseous hydrogen and is carried out in a dipolar aprotic solvent (DMSO) and under mild conditions (Brunner and Leitner, 1988). The oxidative addition of formic acid (the hydrogen donor) to complex (227) is followed by the rate limiting decarboxylation of the monodentate formate ligand to give the dihydrido complex shown, probably by β-hydride elimination from the formate ligand to the transition metal centre (Strauss *et al.*, 1979)]. The formation of the required optically pure product and the requirement of a closed catalytic cycle, such that (227) is regenerated, imply that more itaconic acid (the prochiral substrate and hydrogen acceptor) (224), then reacts with the dihydrido complex (Brunner *et al.*, 1989). It is believed that the Rh(III) centre found in the procatalyst $RhCl_3.nH_2O$ is reduced to Rh(I) by formic acid, in the presence of a phosphane (Brunner *et al.*, 1989). The role of the complex with rhodium in oxidation state one, is believed to be two fold: catalysis of the decomposition of formic acid to



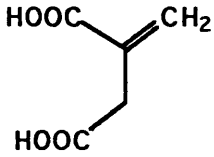
(221a)



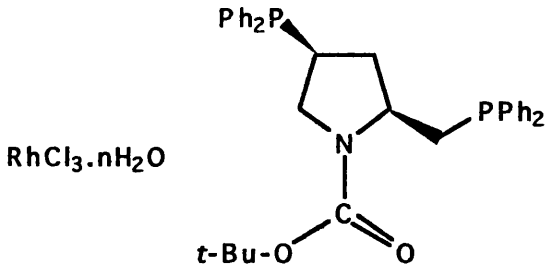
(221b)



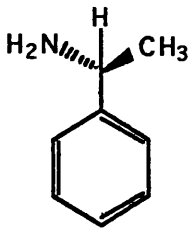
(222) Natural *S*
(223) Synthetic *S*



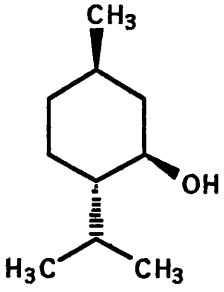
(224)



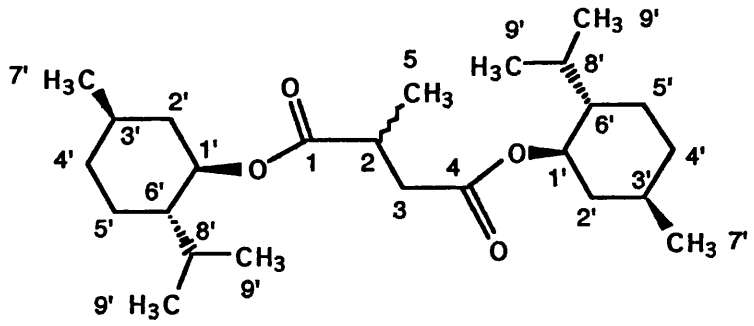
(225)



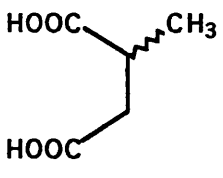
(226)



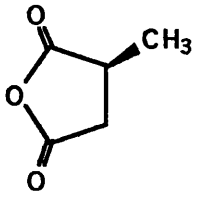
(229)



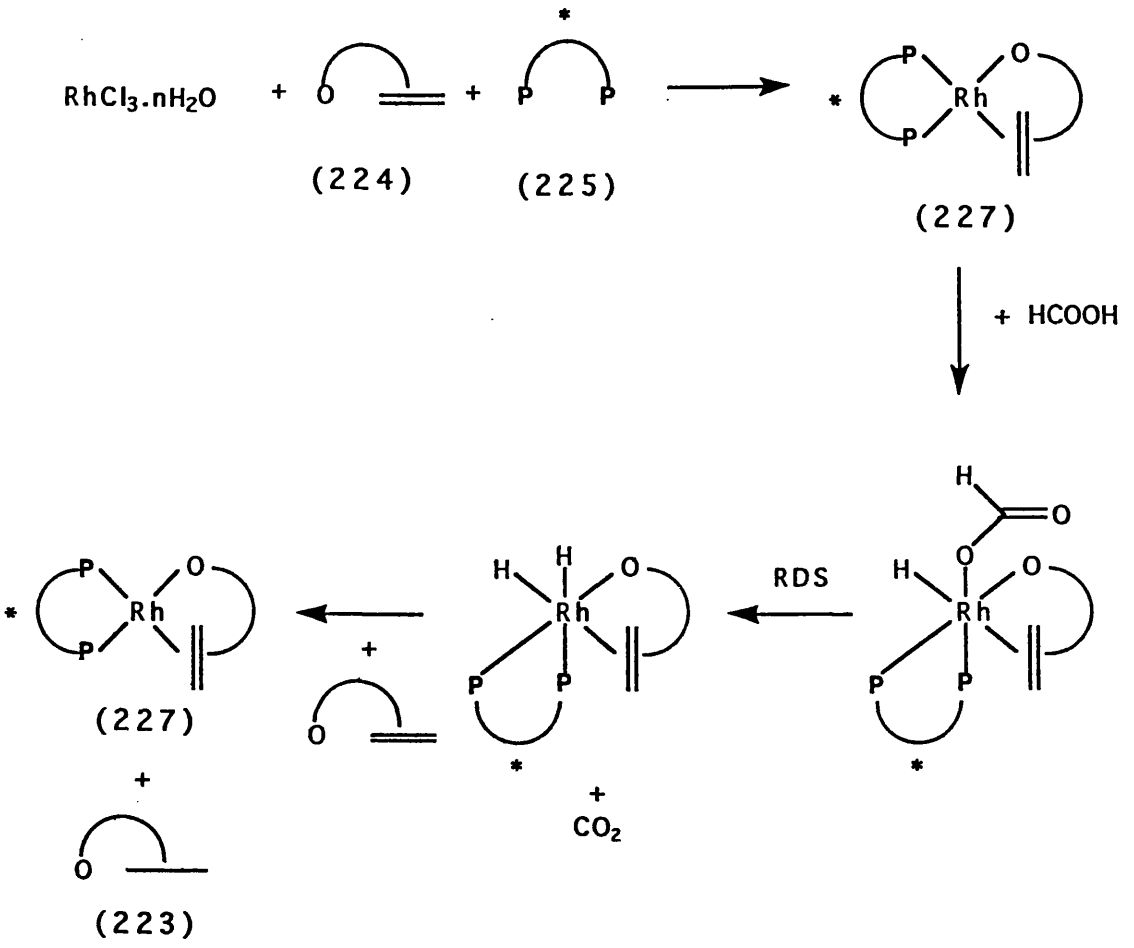
(228) Natural 2*S*
(230) Synthetic 2*S*
(231) 2*RS*



(232)



(233)



hydrogen and carbon dioxide (Strauss *et al.*, 1979) and catalysis of the hydrogenation of the carbon-carbon double-bond containing substrate (Ojima *et al.*, 1980).

The slow rotation of the *tert*-butoxycarbonyl group around the N-C=O bond in chiral phosphane BPPM, which is characteristic of an amide or a carbamate structure is fixed by the bidentate complexation of the substrate and is referred to as the "induced-fit" action of the chiral complex (Ojima *et al.*, 1980). Thus, the chiral recognition is believed to take place in the olefin complexation step and in particular, it is considered that the existence of intermolecular hydrogen bonding may affect the stereoselectivity of the asymmetric hydrogenation of itaconic acid (Ojima *et al.*, 1980). Several seven-membered chelate ring complexes have been proposed and are considered favourable over five-membered ring forming ligands, perhaps due to the lack of readiness to act as so-called "dangling ligands" and thus make coordination sites free at the metal atom (Ojima *et al.*, 1980, Brunner *et al.*, 1988 and Brunner and Leitner, 1988).

The influence of the amine component, (*S*)-1-phenylethylamine (226), on this highly enantioselective catalytic hydrogenation is not certain but a molar ratio of 5:2 formic acid:amine was used, following the work of Brunner *et al.* (1989).

The ^{1}H NMR spectrum (270MHz) of synthetic *S*-(-)-methylsuccinic acid is shown in Figure (21) (B). The melting point and more importantly the optical rotation were in agreement with literature values, confirming the configuration of both the natural and synthetic diacids as *S* (See Section 2.3.2.9).

The *l*-dimenthyl ester of natural *S*-(-)-methylsuccinic acid (228) was prepared to study its ^{13}C NMR spectrum and thus, following the success of the NMR studies of Tsubokura *et al.* (1992), determine the chirality of the methylsuccinimide moiety. The synthesis was achieved by converting diacid (222), obtained from (1), into (228) using *l*-menthol (229) (Furuta *et al.*, 1989) (See Section 2.3.2.10). The ^{1}H NMR spectrum (270MHz) is shown in Figure (22). In the ^{13}C NMR spectrum (100.4MHz) [Figure (23) (A and B)] two signals at 37.92 and 37.85ppm (Δ = 0.07ppm) are observed for the carbon atom at the 3-position of natural *l*-dimenthyl methylsuccinate (C-3). From the intensity ratio of 11:1 for the peaks for this key methylene carbon, that is, α to the chiral carbon, the enantiomeric excess (e.e.) was determined to be 92%. In the literature, the *S*-form and *R*-form have been reported at 38.03 and 37.95ppm, respectively, suggesting that our natural methylsuccinic moiety has an *S*-methyl group (Tsubokura *et al.*, 1992). C-8' could not be confidently assigned (either 31.4 and/or 26.2ppm). The broad peak at 22.0ppm is assigned as two of the geminal methyl groups, C-9'.

The preparation of *l*-dimenthyl *S*-(-)-methylsuccinate (230) from synthetic *S*-(-)-methylsuccinic acid (223) aimed to provide unequivocal evidence for the methylsuccinimide absolute stereochemistry as *S* in MLA (1) (Tsubokura *et al.*, 1992) (See Section 2.3.2.11). This was successfully achieved by studying the ^{13}C NMR spectrum (67.8MHz) [Figure (23) (C)], which showed a signal at

Figure (20) ^{1}H NMR (270MHz) Spectrum of *N*-[2-(*S*)-Methylsuccinyl]anthranilic Acid (221a) and *N*-[3-(*S*)-Methylsuccinyl]anthranilic Acid (221b) in D_2O

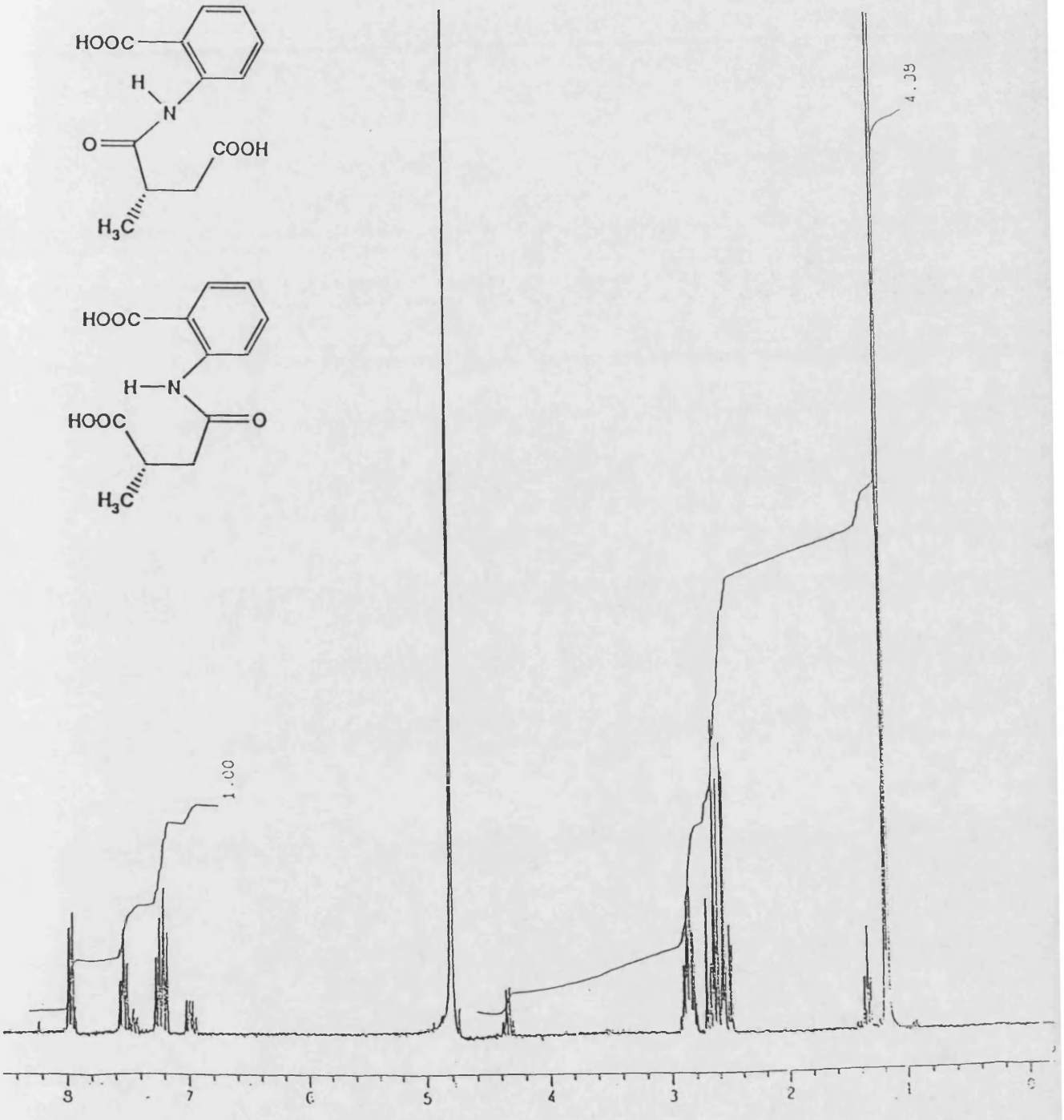
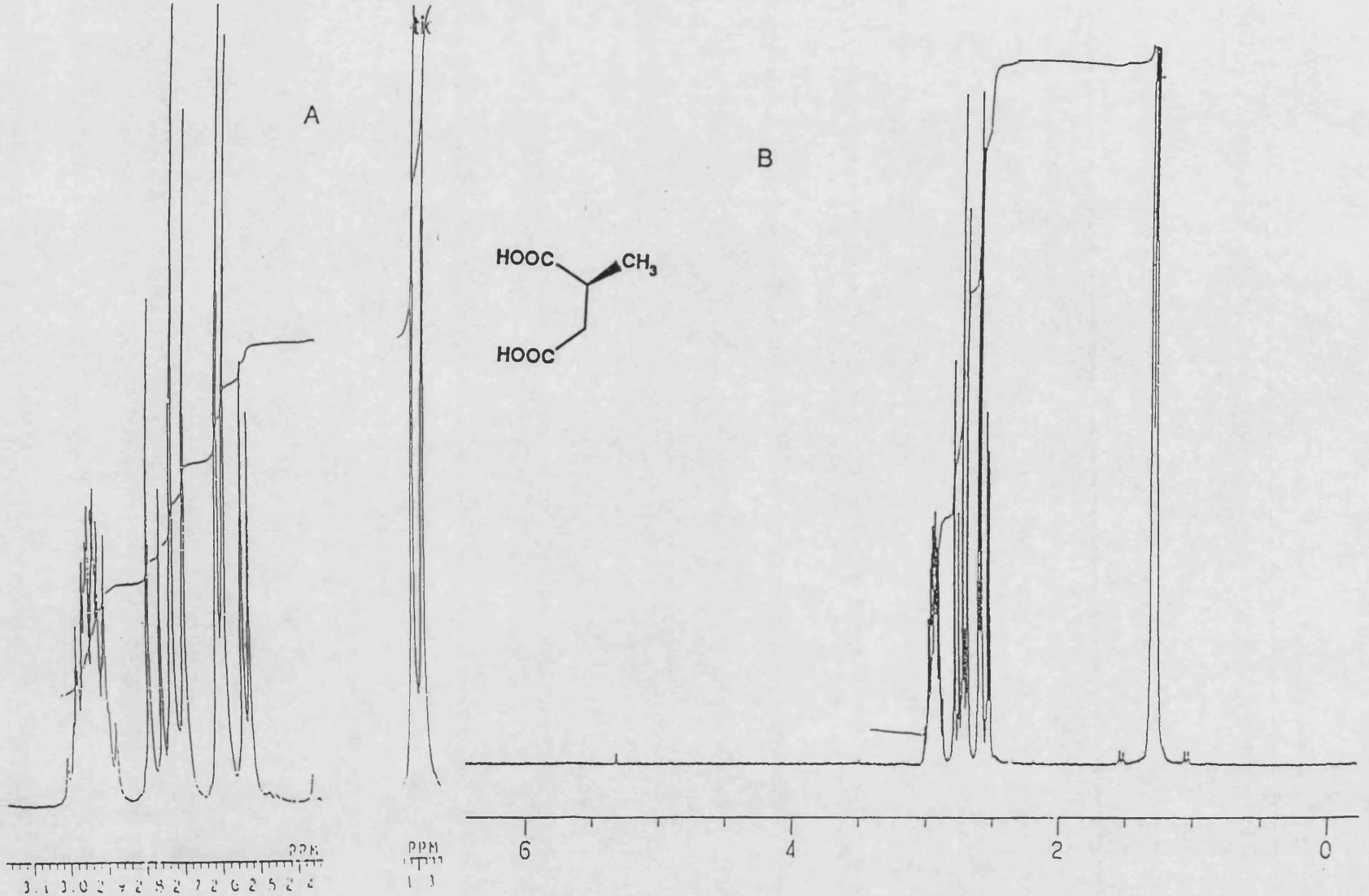


Figure (21) Expansion of ^1H NMR (270MHz) Spectrum of Natural *S*-(-)-Methylsuccinic Acid (222) in $CDCl_3$ (A) and ^1H NMR (270MHz) Spectrum of Synthetic *S*-(-)-Methylsuccinic Acid (223) in $CDCl_3$ (B)



37.94ppm for the methylene carbon, that is, C-3. The e.e. was determined to be 97% based on the ^{13}C NMR analysis, as before.

The closeness of these values clearly gives rise to some ambiguity since the chemical shift obtained for the synthetic *S*-isomer is the same as that assigned to the natural *R*-isomer. In order to resolve this conflict, the *l*-dimenthyl ester (231) was prepared from racemic diacid (232) (See Section 2.3.2.12). The ^{13}C NMR spectrum (100.4MHz) [Figure (23) (D)] obtained, was more complicated than for the single enantiomer, but displayed two signals (which could clearly be resolved at 100.4MHz) for the methylene carbon (C-3): For the *R*-enantiomer δ = 37.82ppm was seen and for the *S*-enantiomer δ = 37.88ppm (Δ = 0.06ppm) was observed. The chiral carbon signal itself was not resolved into two signals (36.07ppm). The ^{13}C NMR spectrum for *l*-dimenthyl (*RS*)-methylsuccinate (231) also revealed two signals for each of C-1, C-4, and C-5. The peak at 36.1ppm for C-2 was broad. However, only two peaks were seen for C-1'-C-8' and four peaks for C-9', as for the single isomer derivatives.

Furthermore, close inspection of the ^{13}C NMR spectrum, after dilution of racemic *l*-dimenthyl ester (231) with one molar equivalent of synthetic *l*-dimenthyl *S*-(-)-methylsuccinate (230), revealed a signal at 37.88ppm with approximately half the intensity of the higher frequency signal (seen at 37.96ppm) (67.8MHz). Taken in isolation the chemical shifts for the two enantiomers are so close (Δ = 0.08ppm) as to be ambiguous, but when used in conjunction with the other chemical shift data obtained, this proves to be the critical experiment confirming that the downfield peak is due to the *S*-isomer.

This ^{13}C NMR spectroscopic procedure, by which the *l*-dimenthyl ester (228) of natural methylsuccinic acid (222) has been shown to be the *S*-enantiomer, will be applicable to the analysis of other methylsuccinimides or anhydrides, including the half-ester amides and the bis-amides which may be artifacts of norditerpenoid alkaloid isolation.

Synthetic diacid (223) was converted into the corresponding *S*-(-)-methylsuccinic anhydride (233) using acetyl chloride (Lowe and Potter, 1980) (See Section 2.3.2.13). An expansion of the ^1H NMR spectrum (270MHz) of the synthetic *S*-(-)-methylsuccinic anhydride produced is shown in Figure (24). The optical rotation {$[\alpha]_D$ = -36.3° (c = 3.5, dioxane at 23°C)} confirmed the stereochemistry as *S*, based on the literature for *R*-(+)-methylsuccinic anhydride {lit. $[\alpha]_D$ = +32.6° (c = 15.0, dioxane at 20°C) (Berner and Leonardsen, 1939)}.

Figure (22) ^1H NMR Spectrum (270MHz) of Natural *l*-Dimenthyl *S*-(-)-Methylsuccinate (228) in $CDCl_3$

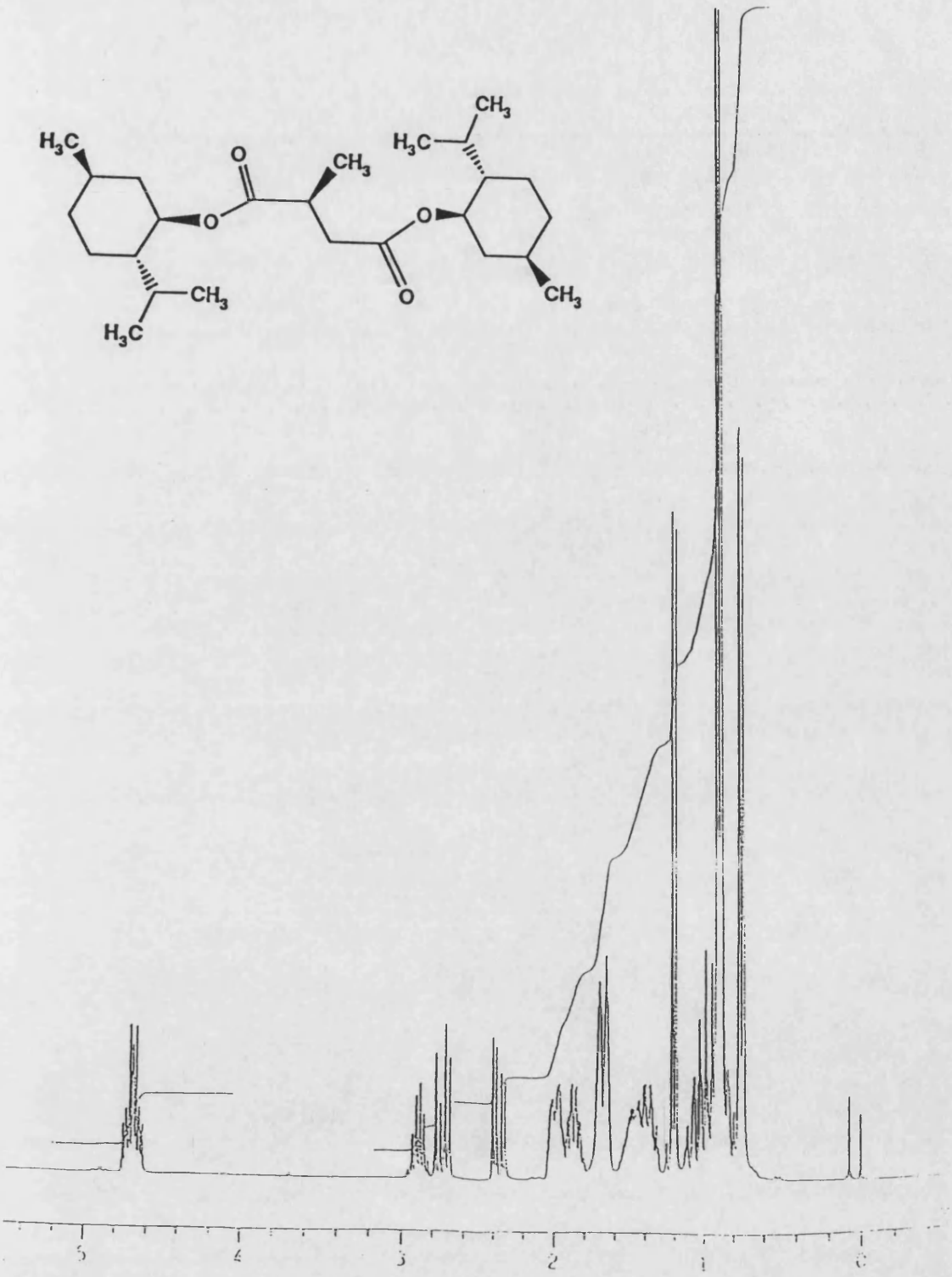


Figure (23) ^{13}C NMR (100.4MHz) Spectrum of Natural *l*-Dimenthyl *S*-(-)-Methylsuccinate (228) in $CDCl_3$ **(A)** and Expansions of ^{13}C NMR Spectra of Natural *l*-Dimenthyl *S*-(-)-Methylsuccinate (228) in $CDCl_3$ (100.4MHz) **(B)**, Synthetic *l*-Dimenthyl *S*-(-)-Methylsuccinate (230) in $CDCl_3$ (67.8MHz) **(C)**, and *l*-Dimenthyl (*RS*)-Methylsuccinate (231) in $CDCl_3$ (100.4MHz) **(D)**

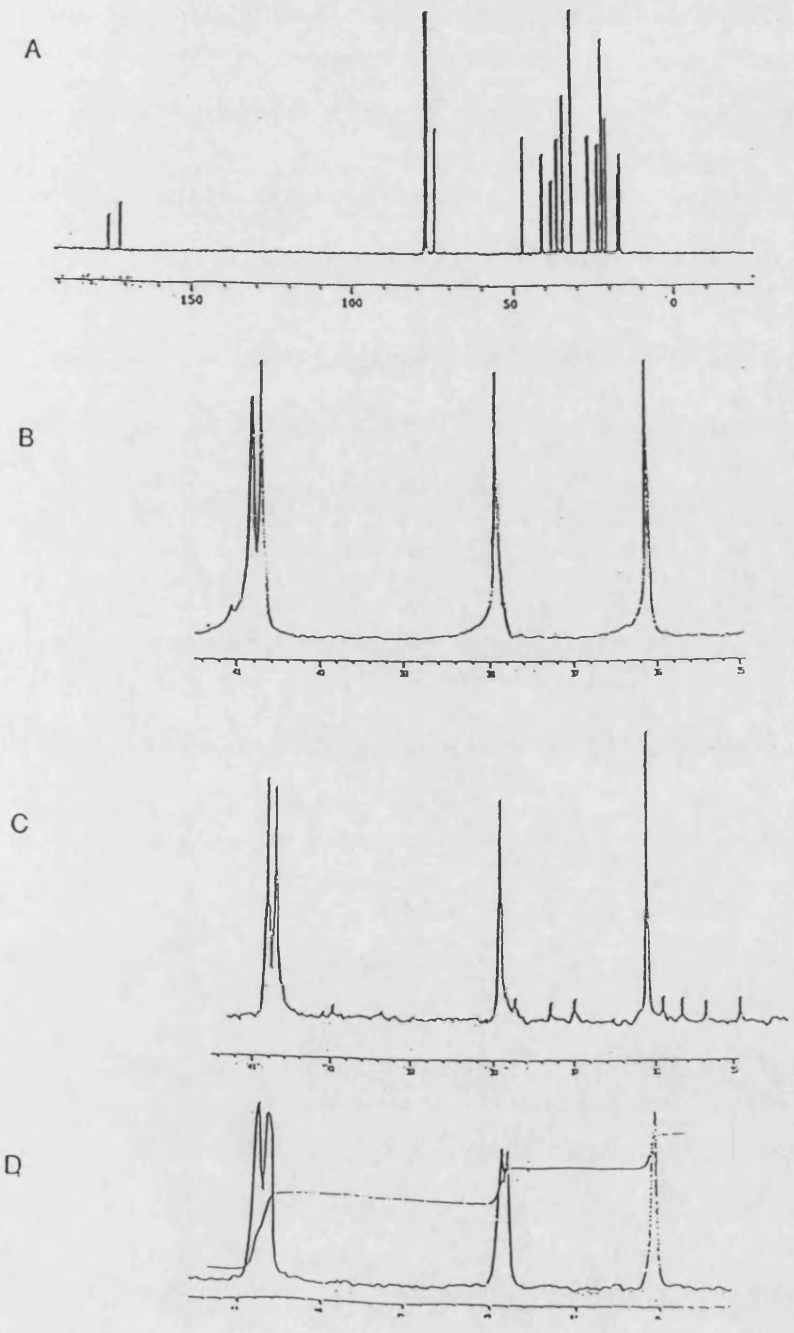
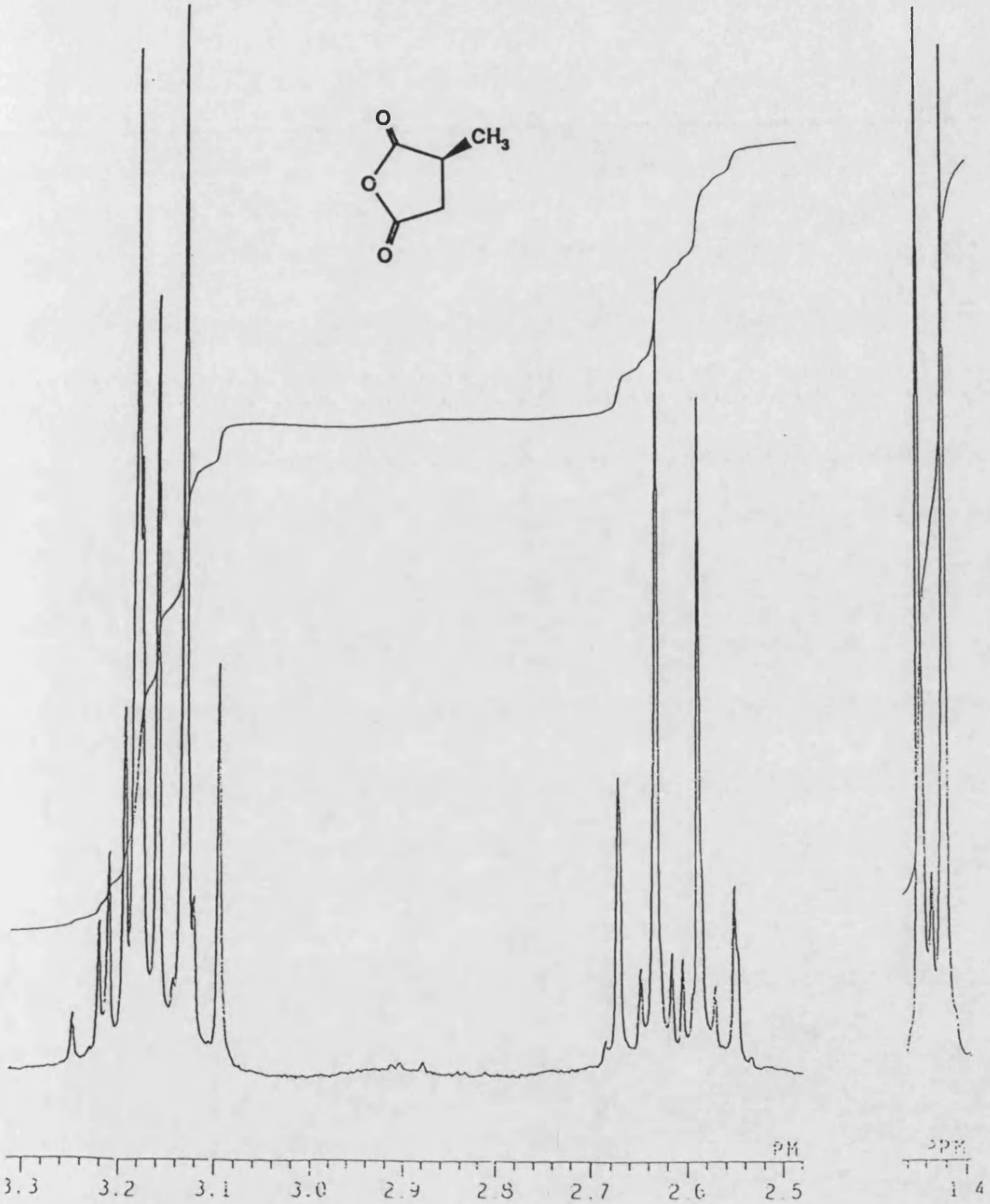


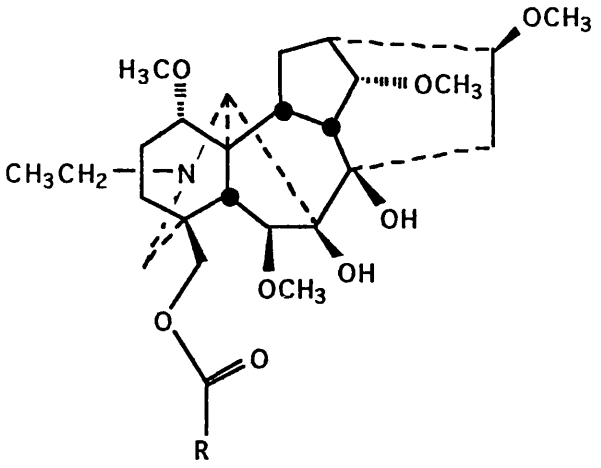
Figure (24) Expansion of ^1H NMR (270MHz) Spectrum of Synthetic *S*-(-)-Methylsuccinic Anhydride (233) in $CDCl_3$



2.2.11 Semi-Synthesis of Lycoctonine, Inuline and MLA

Lycoctonine (2) is a key intermediate in the present studies since its acylation allows access to semi-synthetic norditerpenoid alkaloids, for example, the esters inuline (40), benzoyllycoctonine (234), delsemine (62a) and (62b) (Pelletier and Bhattacharyya, 1977), the half-ester amides (235a) and (235b), the half-acid amides (236a) and (236b), and MLA (1). The half-ester amides (235a) and (235b) may be natural products *per se* or might be artifacts of alcoholysis of (1) on isolation (Mashkovsky and Churyukanov, 1986 and Pelletier *et al.*, 1986a).

Several research groups are actively studying the structure-activity relationships (SAR) of modified anthranilate esters of lycoctonine (2) at C-18, due to the potent pharmacological activity, in particular, of MLA (1) (Kukel and Jennings, 1994). Semi-synthetic studies to date have included the preparation of related esters, structures (237) to (244) (Pelletier and Ross, 1990). However, Cook and Beath (1952) found standard acylation procedures to be problematical. There are many reports of acylation at other positions around the norditerpenoid skeleton of different alkaloids in the literature, for example at C-3, C-13, and C-15 of aconitine (5) (Ross and Pelletier, 1991), at C-1 of delphisine (245) and neoline (246) (Ross and Pelletier, 1988), and at C-13 of delphinine (22) (Pelletier and Ross, 1990), but in many cases there is more than one product. Ross and Pelletier (1991) reported the acylation of *N*-deacetyllappaconitine (247) on the aniline nitrogen atom with a variety of aliphatic and aromatic carboxylic acids in order to increase the lipophilicity of these potential insecticides.



R = C_6H_5 (234)

R = CH_3 (237)

R = C_6H_4-4-OCH_3 (238)

R = C_6H_4-2-OCH_3 (239)

R = C_6H_4-4-NO_2 (240)

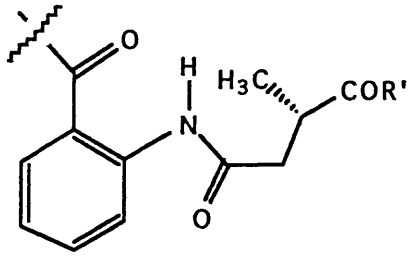
R = C_6H_2-3,4,5-$(OCH_3)_3$ (241)

R = $(CH_2)_{16}CH_3$ (242)

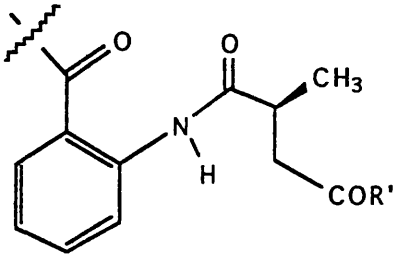
R = $(CH_2)_{10}CH_3$ (243)

R = $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$ (244)

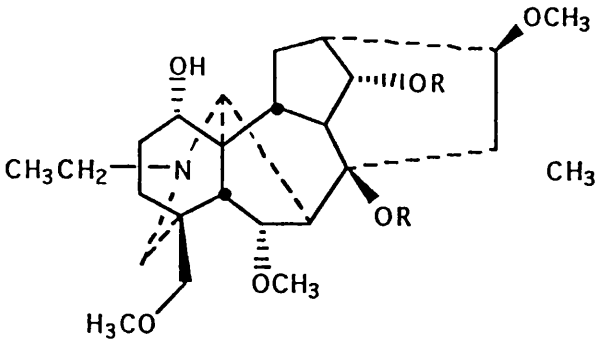
R =



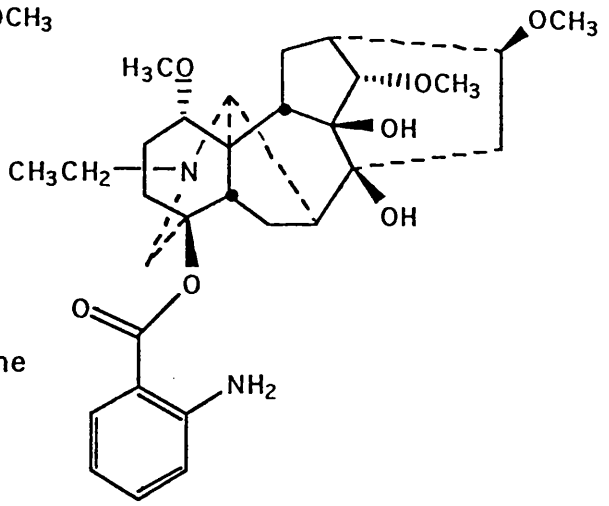
R' = NH_2 (62a)
R' = OCH_3/OCH_2CH_3 (235a)
R' = OH (236a)



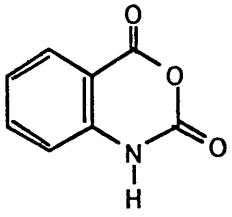
R' = NH_2 (62b)
R' = OCH_3/OCH_2CH_3 (235b)
R' = OH (236b)



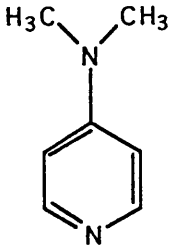
(245) R = Ac, Delphisine
(246) R = H, Neoline



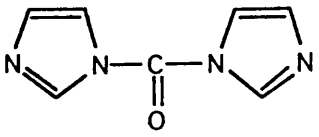
(247) *N*-Deacetyllappaconitine



(248)



(249)



(251)

There is, however, no literature precedent for the facile introduction of the anthranoylmethylsuccinimide moiety found in MLA (1). Thus, we needed to obtain pure lycoctonine (2) in sufficient quantity, by the saponification of MLA (1), to be able to develop protocols for the introduction of the anthranilate ester and for the subsequent addition of the homochiral succinimide moiety to give semi-synthetic MLA. It was important to consider the general application of these methods in natural product synthesis, for example, anhweidelphinine (33) and nudicauline (37).

Ester (1) was saponified with aqueous sodium hydroxide solution to afford the parent alcohol lycoctonine (2) and the aromatic half-acid amides (221a) and (221b), as a mixture of isomers (Manske, 1938 and Pelletier *et al.*, 1980) (See Section 2.3.2.7). The NMR data obtained for lycoctonine (2) can be found earlier, in Section 2.2.6. Cookson *et al.* (1954) reported the hydrolysis of MLA with acid, to give lycoctonine, *S*-(-)-methylsuccinic acid and anthranilic acid. Milder acid hydrolysis of MLA (1) gives inuline (40) (Goodson, 1943).

The synthesis of inuline (40) from lycoctonine (2) was envisaged. We had anticipated that there could be problems attempting to acylate with the zwitterion anthranilic acid and also with bulky *N*-protecting groups typically used in amino acid chemistry [for example, *t*-butoxycarbonyl (*t*-BOC) or carbobenzoxy (CBZ)/benzoxycarbonyl] in the *ortho* position of the phenyl ring. These problems were, therefore, circumvented by the use of isatoic anhydride (248) which acylates to introduce the anthranilate ester with the loss of one equivalent of carbon dioxide (Staiger and Miller, 1959) (See Section 2.3.2.14). The reaction of isatoic anhydride with a variety of alcohols and catalyzed by 4-(*N,N*-dimethylamino)pyridine (DMAP) (249), to give anthranilates will be discussed more fully in Chapter 4. This esterification of lycoctonine (2) is the first semi-synthesis of natural product (40) (Shamma *et al.*, 1979). The primary alcohol of (2) at C-18 acylated preferentially over the tertiary alcohols at C-7 and C-8. Secondary alcohols are known to be sluggish to react with (248) and tertiary alcohols are unreactive (Staiger and Miller, 1959). However, it was important to recognize that this nucleophilic alcohol is associated with a neopentyl-like motif and this bulky substituent may affect esterification. The NMR studies of this anthranoyl ester (40), which has been isolated from a number of *Delphinium* species and was obtained by Goodson (1943) by the acidic hydrolysis of MLA (1), are shown earlier, in Section 2.2.7.

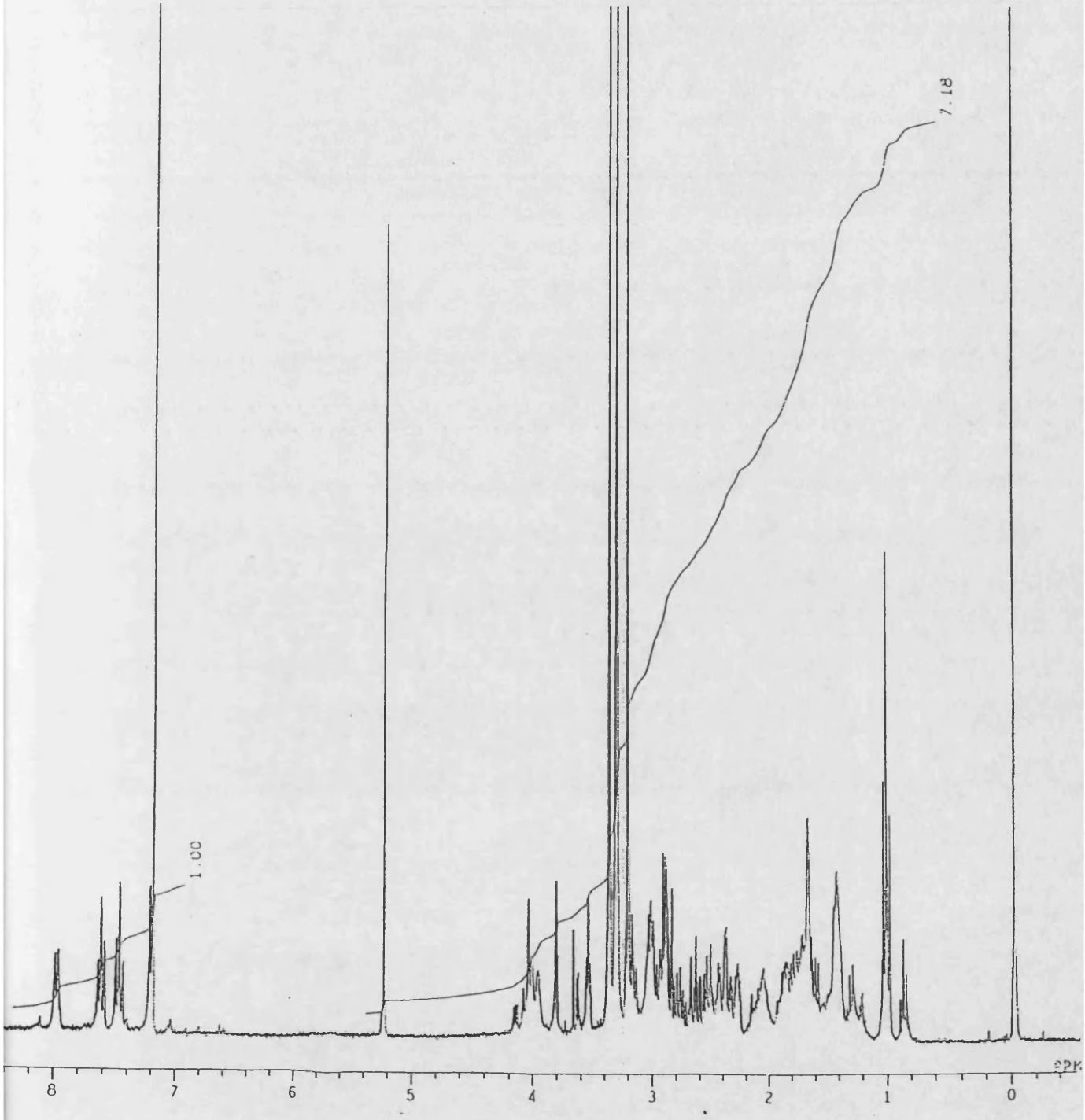
The half-acid amides (236a) and (236b) were synthesized, but not isolated, by treatment of inuline (40) with synthetic *S*-(-)-methylsuccinic anhydride (233) (in dichloromethane) (See earlier in Section 2.2.10 and later in Section 2.3.2.13). The mixture (236a) and (236b) was homogeneous by TLC (R_f = 0.46, 1:1 methanol-dichloromethane). By TLC, all the inuline had reacted after 40hours. Closure of the succinimide ring of the mixture (236a) and (236b) gave (250) and was carried out using 1,1'-carbonyldiimidazole (251) (CDI). The preparation of cyclic imides [succinimides (Flitsch and Ruβkamp, 1984), maleiimides (Garner *et al.*, 1991, Balasubramaniyan and Argade, 1987 and 1988), and phthalimides (Bose *et al.*, 1958, Hoffmann and Schiff-Shenhav, 1962, Kidd and

King, 1948)] and possible problems associated with dehydration reactions of this type (Ito *et al.*, 1991) described in the literature will be discussed more fully in Chapter 4.

The product (250) was found to be identical in all chromatographic and spectroscopic respects with the natural product (1) (See earlier in Section 2.2.5 and later in Section 2.3.2.15). Figure (25) shows the ^1H NMR (270MHz) spectrum of semi-synthetic MLA (250).

Thus, successful semi-synthetic methods have been utilized to obtain MLA (1) from inuline (40), by regiospecific acylation of lycoctonine (2) and incorporation of the *S*-enantiomer of methylsuccinic anhydride (233).

Figure (25) ^{1}H NMR (270MHz) Spectrum of Semi-Synthetic MLA (250) in $CDCl_3$



2.3 EXPERIMENTAL

2.3.1 Instrumentation and Experimental Techniques

2.3.1.1 Solvents and Reagents

Solvents and reagents were dried and purified prior to use according to the procedures described in the "Purification of Laboratory Chemicals" (Perrin and Armarego, 1988). Anhydrous dichloromethane was obtained by distillation from calcium hydride. *N,N*-Dimethylformamide was stored over freshly activated 4Å molecular sieves. Water refers to distilled water.

Chemicals and reagents were routinely purchased from Aldrich, Fisons, Fluka, Lancaster, and Sigma. The seeds of Garden Hybrid *Delphinium* were a gift from the Blackmore and Langdon Ltd.

Moisture sensitive reactions were performed in dry glassware (oven 120°C overnight and/or flame drying under a positive flow of nitrogen). Syringes and stirrer bars were dried and stored over anhydrous calcium chloride.

Solvents were removed with a Buchi rotary evaporator using a water aspirator or a vacuum pump as required and a variable temperature water bath.

The seeds of Garden Hybrid *Delphinium* were crushed using a coffee grinder.

2.3.1.2 Chromatography

Thin layer chromatography (TLC) was used routinely to monitor the progress of reactions and purity of compounds. TLC was performed on pre-coated plates (Merck TLC aluminium sheets silica 60 F_{254}, Art no. 5554). Products were visualized, when possible, by short wavelength (254nm) ultraviolet light and by treatment with iodine vapour, a solution of bromocresol green (0.04g in 100cm^3 ethanol and 0.1M sodium hydroxide until the blue colour just appears), or a solution of ninhydrin (0.3g in *n*-butanol 100cm^3 and 3cm^3 acetic acid). Alternatively, when applicable, Dragendorff Munier spray was used for the visualization of alkaloids (the reagent was prepared from 1.7g basic bismuth nitrate and 20g tartaric acid in 80cm^3 water-16g potassium iodide in 40cm^3 water-10g tartaric acid in 50cm^3 water, 1:1:20). Medium pressure column chromatography was carried out according to the method developed by Still *et al.* (1978) using, unless otherwise stated, Sorbsil C60-H silica gel (40-60μm), purchased from Prolabo, Eccles, Manchester. Columns were packed as a slurry in the eluting solvent. Pressure was applied to the column *via* a pair of small hand bellows. Centrifugally accelerated thin layer chromatography was performed using a Harrison Research Model 7924T chromatotron.

2.3.1.3 Analysis and Spectroscopy

^{1}H NMR spectra were recorded at 90, 270 or 400MHz using a Jeol FX90Q, a Jeol GX270 or a Jeol GX400 spectrometer, respectively. ^{13}C NMR were recorded at 67.8 (FX90Q), 90.6 (GX270) or 100.4MHz (GX400), employing 90° and 135° DEPT pulse sequences to aid multiplicity determinations. Chemical shifts (δ) are expressed in parts per million downfield from an internal standard tetramethylsilane (TMS). Coupling constants (*J*) are expressed in Hertz (Hz). Multiplicities are denoted by s - singlet, d - doublet, t - triplet, q - quartet, quin - quintet, and m - multiplet. The abbreviation br is used to indicate significant

broadening due to rapid exchange or unresolved fine coupling. The format used for reporting ^{1}H NMR spectra is: chemical shift δ (integration, multiplicity, coupling constant *J* Hz, assignment). COSY, long-range COSY, phase-sensitive NOESY, HETCOR, and COLOC spectra were recorded using a Jeol GX400 spectrometer and used to confirm proton assignments where required (Croasmun and Carlson, 1987).

Low resolution mass spectra (MS) were recorded using a VG analytical 7070E instrument with a VG 2000 data system. Electron impact (EI) spectra were produced using an ionizing potential of 70eV. Chemical ionization (CI) was performed using *iso*-butane as reagent gas, and +ve and -ve FAB was performed using 3-nitrobenzyl alcohol or a glycerol matrix.

Melting points (Mp) were determined using a Reichert-Jung Thermo Galen Kopfler block and are uncorrected.

Elemental microanalyses were carried out using a Carla Erba 1106 Elemental Analyser by Mr Carver and the University of Bath microanalytical service.

X-Ray crystallographic investigations were carried out at room temperature using a CAD-4 automatic four-circle diffractometer using Mo-K_α radiation, in the range $2 \leq \theta \leq 22°$, operated by Dr Mahon at the University of Bath.

Optical rotations were measured using an Optical Activity Ltd. polarimeter.

2.3.2 Experimental Work

2.3.2.1 Extraction of Garden Hybrid *Delphinium* Seeds

Seeds of Garden Hybrid *Delphinium* (12g) were ground and defatted with redistilled hexane (210cm^3) in a Soxhlet extractor (presoak of 21h). The seeds were then extracted with redistilled chloroform (180cm^3) (presoak of 21h). The extract was concentrated under reduced pressure (to 50cm^3) and then extracted with aqueous sulfuric acid solution (0.75M, 65cm^3). The acidic layer was basified to pH 10 (saturated aqueous sodium carbonate solution) and then extracted with redistilled diethyl ether (3 x 50cm^3). Drying (Na_2SO_4) and evaporation under reduced pressure of the combined organic layers gave crude alkaloidal material as an off-white foam (147mg, 1.22% weight of seeds taken); TLC (cyclohexane-chloroform-diethylamine, 5:4:1 detection by Dragendorff Munier spray) showed 3 main bands.

The success of this pilot run was followed by a large scale seed extraction. Thus, seeds of Garden Hybrid *Delphinium* (600g) were ground and defatted with redistilled hexane (3.5dm^3) in a Soxhlet extractor (presoak of 20h). The seeds were then extracted with redistilled chloroform (3.0dm^3) (presoak of 65h). The extract was concentrated under reduced pressure (to 500cm^3) and then extracted with aqueous sulfuric acid solution (0.1M, 500cm^3). The acidic layer was basified to pH 10 (saturated aqueous sodium carbonate solution) and then extracted with redistilled diethyl ether (3 x 350cm^3). Drying (Na_2SO_4) and evaporation under reduced pressure of the combined organic layers gave crude alkaloidal material as an off-white foam (7.48g, 1.25% weight of seeds taken). Thus, the 12g and 600g scale seed extractions were performed with essentially equal efficiency.

[See Section 2.2.1]

2.3.2.2 Purification of Crude Alkaloidal Material by Preparative Thin Layer Chromatography

Crude alkaloidal material (70mg) was purified by preparative thin layer chromatography (pTLC) (silica G, 2mm) (by evenly applying 10mg in approximately 50μl chloroform to each 20x20cm plate, taking care not to damage the silica). Elution was performed using cyclohexane-chloroform-diethylamine 5:4:1. The separation was visualized with Dragendorff Munier reagent (by covering the plate except for two strips at the edges and spraying the exposed strips). Three partially separated bands of compounds were scraped from the plate with a spatula and collected. 3:1 Chloroform-methanol was used to extract the alkaloid fractions from the silica. The solvent was removed under reduced pressure and the three fractions were redissolved in diethyl ether (as a precaution against dissolved compounds from the silica). Filtration and evaporation gave three fractions. Fraction 1 (13mg): one major alkaloidal component shown to be delpheline (219) with $R_f = 0.45$ (cyclohexane-chloroform-diethylamine 5:4:1, detection by Dragendorff Munier spray). Fraction 2 (14mg): one major alkaloidal component shown to be MLA (1) with $R_f = 0.30$ (cyclohexane-chloroform-diethylamine 5:4:1, detection by Dragendorff Munier spray). Fraction 3 (7mg): a mixture including the alkaloid found in Fraction 2, MLA (1) and traces of more polar alkaloids [minimum of six components by TLC (cyclohexane-chloroform-diethylamine 5:4:1, detection by Dragendorff Munier spray)].

[See Section 2.2.2]

2.3.2.3 Purification of Crude Alkaloidal Material by Centrifugal Chromatography

Crude alkaloidal material (200mg) (dissolved in 1ml chloroform), was purified by centrifugal chromatography (CC) over silica (2mm). Elution was performed using cyclohexane-chloroform-diethylamine 5:4:1. The appropriate fractions were combined and evaporated under reduced pressure following TLC analysis (cyclohexane-chloroform-diethylamine 5:4:1, detection by Dragendorff Munier spray) to give six groups of fractions. Group 1 (3mg, Fraction 9): non-alkaloidal material and a trace of an alkaloidal component shown to be delpheline (219) with $R_f = 0.45$. Group 2 (38mg, Fractions 10 and 11): a mixture of predominantly the alkaloid found in Group 1 and traces of two more polar alkaloids (by TLC). Group 3 (59mg, Fractions 12 and 13): a mixture of two alkaloids, one component shown to be the same major alkaloidal component as Group 1 (by TLC) and the other component shown to be MLA (1) with $R_f = 0.30$. Group 4 (30mg, Fractions 14-16): a mixture of predominantly the alkaloid found in Group 3, MLA (1) and traces of two more polar alkaloids (by TLC). Group 5 (38mg, Fractions 17-35): a mixture including the alkaloid found in Group 3, MLA (1), a more polar alkaloid, and traces of two more polar alkaloids (by TLC). Group 6 (29mg, Fractions 36-44): a mixture of polar alkaloids (minimum of six components by TLC).

[See Section 2.2.2]

2.3.2.4 Purification of Crude Alkaloidal Material by Vacuum Liquid Chromatography

Crude alkaloidal material (992mg), from a large scale seed extraction, was purified by vacuum liquid chromatography (VLC) over alumina. Elution was performed using a stepped gradient of mixtures of hexane, diethyl ether, and methanol, in order of increasing polarity. The appropriate fractions were combined and evaporated under reduced pressure following TLC analysis (cyclohexane-chloroform-diethylamine 5:4:1, detection by Dragendorff Munier

spray) to give six groups of fractions. Group 1 (121mg, Fractions 1-7, eluted with up to 60% diethyl ether-hexane): one major alkaloidal component shown to be delpheline (219) with R_f = 0.45 and also non-alkaloidal material. Group 2 (52mg, Fractions 8-12, eluted with up to 2.5% methanol-diethyl ether): same major component as Group 1 (by TLC). Group 3 (379mg, Fractions 13 and 14, eluted with up to 7.5% methanol-diethyl ether): one major alkaloidal component shown to be MLA (1) with R_f = 0.30. Group 4 (59mg, Fraction 15, eluted with up to 10% methanol-diethyl ether): a mixture of predominantly the alkaloid found in Group 3, MLA (1) and a trace of a more polar alkaloid (by TLC). Group 5 (164mg, Fractions 16-20, eluted with up to 30% methanol-diethyl ether): a mixture of polar alkaloids (minimum of seven components by TLC). Group 6 (30mg, Fractions 21 and 22, eluted with up to 40% methanol-diethyl ether): a mixture of two highly polar alkaloids (by TLC).

[See Section 2.2.2]

2.3.2.5 Recrystallisation and Characterization of Delpheline (219) (Chemical Abstracts Registry Number [509-28-4]).

Crude alkaloid (219) obtained by VLC (67mg) was dissolved in a minimum volume of hot (~65°C) ethanol-hexane (1:1) and after cooling for 7 days white crystals separated out. Filtration and washing with cold (5°C) hexane gave (219) as white crystalline material (39mg, 59%). Mp 216-218°C [lit. Mp 217.5-219.5°C (Pelletier *et al.*, 1989b)]; TLC (cyclohexane-chloroform-diethylamine 5:4:1, detection by Dragendorff Munier spray) R_f = 0.45. After detailed comparison with and reassignment of published spectroscopic data (^1H and ^{13}C NMR) (Bando *et al.*, 1989 and Joshi *et al.*, 1991), the alkaloid (219) was identified as delpheline. δ_H ($CDCl_3$)/ppm (400MHz) 5.13 (1H, s, OC*H*β O), 5.05 (1H, s, OC*H*α O), 4.19 (1H, s, H-6), 3.71-3.64 (1H, m, H-14), 3.67-3.60 (1H, m, H-9), 3.43 [3H, s, C(14)OC*H*$_3$], 3.35 [3H, s, C(16)OC*H*$_3$], 3.34 [1H, s, C(6)OH], 3.26 [3H, s, C(1)OC*H*$_3$], 3.25-3.19 (1H, m, H-16), 3.08 (1H, br s, H-17), 3.02 (1H, dd, *J* 9.8 and 7.3, H-β-1), 2.77 (1H, dq, *J* 7.1 and 12.0, 1H of NC*H*$_2$ CH_3), 2.69-2.60 (2H, m, H-α-19 and 1H of NC*H*$_2$ CH_3), 2.55 (1H, dd, *J* 14.6 and 4.9,

H-α-12), 2.49 (1H, dd, *J* 14.6 and 8.8, H-α-15), 2.37 (1H, dd, *J* 6.8 and 4.9, H-13), 2.24 (1H, d, *J* 11.7, H-β-19), 2.21-2.08 (2H, m, H-α-2 and H-10), 2.07-1.98 (1H, m, H-β-2), 1.86-1.78 (2H, m, H-β-12 and H-β-15), 1.59 (1H, ddd, *J* 13.1, 4.9, and 2.1, H-α-3), 1.26-1.16 (1H, m, H-β-3), 1.22 (1H, s, H-5), 1.06 (3H, t, *J* 7.1, NCH_2CH_3), and 0.93 (3H, s, H_3-18); δ_C ($CDCl_3$)/ppm (100.4MHz) 92.9 (OCH_2O), 92.7 (C-7), 84.1 (C-8), 83.0 (C-14), 82.7 (C-1), 81.8 (C-16), 79.2 (C-6), 63.6 (C-17), 57.8 [C(14)OCH_3], 57.4 (C-19), 56.6 (C-5), 56.3 [C(16)OCH_3], 55.5 [C(1)OCH_3], 50.6 (NCH_2CH_3), 50.4 (C-11), 47.7 (C-10), 40.3 (C-9), 37.7 (C-13), 36.9 (C-3), 33.8 (C-4), 33.4 (C-15), 28.1 (C-12), 26.7 (C-2), 25.3 (C-18), and 13.8 (NCH_2CH_3); $C_{25}H_{39}NO_6$ requires MW 449 and C, 66.8; H, 8.7; N, 3.1%; Found: m/z (+) FAB 450 (MH^+, 100%), 419 [$(MH-OCH_3)^+$, 30%], (-) FAB 448 (M^--1, 12%) and 418 [$(M-OCH_3)^-$, 100%] and C, 67.0; H, 8.9; N, 3.1%; $[\alpha]_D$ = -27.0° (c = 0.25, ethanol at 25°C) {lit. $[\alpha]_D$ = -26.8° (c = 0.3, ethanol at 26°C) (Goodson, 1943 and Pelletier *et al.*, 1983)}. The crystal data for delpheline (219) was obtained from a crystal of approximate dimensions 0.3 x 0.3 x 0.4mm: unit cell parameters *a* = 12.064 (2), *b* = 13.276 (2), *c* = 14.514 (2)Å, *U* = 2324.5Å^3. For *Z* = 4 the computed density was D_c = 1.28gcm^{-3}. Examination of the systematic extinctions indicated that the orthorhombic crystal belonged to the space group $P2_12_12_1$. Total number of electrons in unit cell, *F*(000) = 976. 2736 reflections were collected of which 1999 were unique with intensities $I \geq 2\sigma(I)$. Data were corrected for Lorentz and polarization effects but not for absorption. The structure was solved by direct methods and refined using the SHELX suite of programs, that is, by full-matrix least-squares refinement, to give final residuals values, after 7 cycles of least-squares of *R* = 0.0438, R_w = 0.0479, for a weighting scheme of $w = 1.9775/[\sigma^2(F) + 0.0001045(F)^2]$. In the final least squares cycles all atoms were allowed to vibrate anisotropically. Hydrogen atoms were included at calculated positions except in the case of C(6)O*H* which was located in an advanced difference Fourier and refined at a fixed distance of 0.98Å from the relevant parent atom.

[See Sections 2.2.3, 2.2.4, 2.2.8 and 2.2.9]

2.3.2.6 Characterization of MLA (1)
(Chemical Abstracts Registry Number [21019-30-7]).

After detailed comparison with published spectroscopic data (^1H and ^{13}C NMR) (Chen and Wu, 1990 and Sun and Benn, 1992), the alkaloid (1) was identified as MLA. TLC (cyclohexane-chloroform-diethylamine 5:4:1, detection by Dragendorff Munier spray) $R_f = 0.30$ (authentic MLA $R_f = 0.31$). δ_H ($CDCl_3$)/ppm (400MHz) 8.05 (1H, dd, *J* 7.6 and 1.6, H-6'), 7.70 (1H, dt, *J* 7.6 and 1.6, H-4'), 7.55 (1H, dt, *J* 7.6 and 1.3, H-5'), 7.30 (1H, dd, *J* 7.6 and 1.3, H-3'), 4.15-4.00 (2H, m, H-β-18 and H-α-18), 3.85 (1H, s, H-6), 3.60 (1H, dd, *J* 4.8 and 4.6, H-14), 3.45 [3H, s, C(6)OC*H*$_3$], 3.42 [3H, s, C(14)OC*H*$_3$], 3.38 [3H, s, C(16)OC*H*$_3$], 3.28 [3H, s, C(1)OC*H*$_3$], 3.24-3.17 (1H, m, H-16), 3.10-3.03 (2H, m, H-9 and H-3"), 3.02-2.96 (1H, m, H-2"), 2.95-2.92 (3H, m, H-β-1, H-17, and 1 of NC*H*$_2$ CH_3), 2.73-2.69 (2H, m, H-α-19 and 1 of NC*H*$_2$ CH_3), 2.64-2.56 (1H, m, H-α-15), 2.56-2.50 (1H, m, H-3"), 2.50-2.45 (1H, m, H-α-12), 2.45-2.38 (1H, m, H-β-19), 2.35-2.31 (1H, m, H-13), 2.20-2.15 (1H, m, H-α-2), 2.15-2.03 (1H, m, H-β-2), 1.98-1.90 (1H, m, H-10), 1.90-1.80 (1H, m, H-β-12), 1.78-1.72 (1H, m, H-α-3), 1.70-1.64 (2H, m, H-5 and H-β-15), 1.58-1.52 (1H, m, H-β-3), 1.47 (3H, br d, *J* 7.8, H_3-5"), and 1.07 (3H, t, *J* 7.0, NCH_2C*H*$_3$); δ_C ($CDCl_3$)/ppm (100.4MHz) 179.8 (C-1"), 175.8 (C-4"), 164.1 (CO), 133.7 (C-4'), 133.1 (C-2'), 131.0 (C-6'), 130.0 (C-3'), 129.4 (C-5'), 126.9 (C-1'), 90.8 (C-6), 88.5 (C-7), 83.9 (C-1), 83.0 (C-14), 82.6 (C-16), 77.5 (C-8), 69.5 (C-18), 64.5 (C-17), 58.1 [C(6)O*C*H_3], 57.8 [C(14)O*C*H_3], 56.3 [C(16)O*C*H_3], 55.8 [C(1)O*C*H_3], 52.4 (C-19), 50.9 (NCH_2CH_3), 50.3 (C-5), 49.1 (C-11), 46.1 (C-10), 43.2 (C-9), 38.0 (C-13), 37.6 (C-4), 37.0 (C-3"), 35.2 (C-2"), 33.6 (C-15), 32.1 (C-3), 28.7 (C-12), 26.1 (C-2), 16.4 (C-5"), and 14.1 (NCH_2CH_3); $C_{37}H_{50}N_2O_{10}$ requires MW 682 and C, 65.1; H, 7.4; N, 4.1%; Found: m/z (+) FAB 683 (MH^+, 100%), 668 [$(MH-CH_3)^+$, 6%], 652 [$(MH-OCH_3)^+$, 4%], 216 ($C_{12}H_{10}NO_3^+$, 12%), (-) FAB 681 (M^--1, 21%), 666 [$(M-CH_3-1)^-$, 100%] and C, 65.0; H, 7.2; N, 4.1%; $[\alpha]_D = +53.4°$ (c = 1.5, ethanol at 25°C) {lit. $[\alpha]_D = +53.7°$ (c = 0.8, methanol at 15°C), $[\alpha]_D = +53.2°$ (c = 2, ethanol at 20°C). and $[\alpha]_D = +49.1°$ (c = 2, ethanol at 22°C) (Goodson, 1943)}.

[See Sections 2.2.5, 2.2.8 and 2.2.9]

2.3.2.7 Hydrolysis of MLA (1) to give Lycoctonine (2), *N*-[2-(*S*)-Methylsuccinyl]anthranilic acid (221a) and *N*-[3-(*S*)-Methylsuccinyl]anthranilic acid (221b) (Chemical Abstracts Registry Number for lycoctonine [26000-17-9]).

To a stirred solution of partially purified MLA (1) (1.00g, 75%, 1.1mmol) in absolute ethanol (redistilled, 15cm^3) at 25°C was added aqueous sodium hydroxide (0.1M, 1.7cm^3, 1.7mmol) in one portion. The resulting pale yellow solution was stirred until completion of the reaction (16h) and then acidified to pH 10 (0.1M aqueous hydrochloric acid solution, ~15cm^3). The ethanolic solution was extracted with dichloromethane (3 x 20cm^3) and the combined organic layers were then washed successively with water (15cm^3) and brine (15cm^3), dried ($MgSO_4$), and concentrated under reduced pressure. The residual yellow oil was purified over silica gel (30g) eluted with 10% methanol in dichloromethane plus ammonia (<1%) and then recrystallized from hot (~65°C) ethanol [filtration and washing with cold (5°C) diethyl ether] to give (2) as white crystalline material (380mg, 74%). Mp 114-117°C [lit. Mp 151-153°C, 112-114°C, 95-97.5°C (Pelletier *et al.*, 1984)]; TLC [cyclohexane-chloroform-diethylamine 5:4:1, detection by Dragendorff Munier spray and by short wavelength (254nm) UV lamp] showed the disappearance of MLA (1) and the appearance of a more polar, non UV-active alkaloid with $R_f = 0.20$. After detailed comparison with published spectroscopic data (^1H and ^{13}C NMR) (Pelletier *et al.*, 1984), the alkaloid (2) was confirmed as lycoctonine. δ_H ($CDCl_3$)/ppm (270MHz) 4.08 [1H, s, C(8)OH], 3.84 (1H, s, H-α-6), 3.68-3.59 (2H, m, H-14 and H-α-18), 3.44 [3H, s, C(6)OC*H*$_3$], 3.41 [3H, s, C(14)OC*H*$_3$], 3.35 (1H, d, *J* 9.4, H-β-18), 3.34 [3H, s, C(16)OC*H*$_3$], 3.25 [3H, s, C(1)OC*H*$_3$], 3.21 (1H, dd, *J* 8.8 and 7.8, H-16), 3.08-3.05 (1H, m, H-13), 2.96-2.88 (3H, m, H-β-1, H-17, and 1 of NC*H*$_2$ CH_3), 2.87-2.75 (1H, m, 1 of NC*H*$_2$ CH_3), 2.63-2.57 (2H, m, H-α-15 and H-α-19), 2.43 (1H, dd, *J* 14.1, 4.7, and 4.7, H-α-12), 2.33 (1H, dd, *J* 4.7, 4.7, and 6.8, H-10), 2.27 (1H, dd, *J* 11.5 and 1.6, H-β-19), 2.20-2.01

(2H, m, H-β-2 and H-α-2), 1.94-1.78 (2H, m, H-5 and H-β-12), 1.75 [1H, br s, C(7)OH], 1.69-1.63 (3H, m, H-α-3, H-9, and H-β-15), 1.59-1.47 [2H, m, H-β-3 and C(18)OH], and 1.04 (3H, t, *J* 7.1, NCH_2CH_3); δ_C ($CDCl_3$)/ppm (90.6MHz) 90.6 (C-6), 88.4 (C-7), 84.2 (C-1), 83.9 (C-14), 82.5 (C-16), 77.4 (C-8), 67.7 (C-18), 64.8 (C-17), 57.9 [C(6)OCH_3], 57.8 [C(14)OCH_3], 56.2 [C(16)OCH_3], 55.8 [C(1)OCH_3], 52.5 (C-19), 51.1 (NCH_2CH_3), 49.5 (C-9), 48.8 (C-11), 46.1 (C-5), 43.2 (C-13), 38.5 (C-4), 38.0 (C-10), 33.5 (C-15), 31.6 (C-3), 28.7 (C-12), 26.1 (C-2), and 14.1 (NCH_2CH_3); $C_{25}H_{41}NO_7$ requires MW 467 and C, 64.2; H, 8.8; N, 3.0%; Found: m/z (+) FAB 468 (MH^+, 100%), 436 [$(M-OCH_3)^+$, 26%], (-) FAB 466 (M^--1, 100%) and C, 64.1; H, 8.8; N, 3.2%; $[\alpha]_D$ = +52.4° (c = 1.5, ethanol at 25°C) {lit. $[\alpha]_D$ = +52.2° (c = 0.8, methanol at 22°C) (Manske, 1938) and $[\alpha]_D$ = +49.64° (c = 9, ethanol at 20°C) (Goodson, 1943)}.

[See Sections 2.2.6, 2.2.8, 2.2.9 and 2.2.11]

The aqueous layer was acidified to pH 3 (0.1M aqueous hydrochloric acid solution, ~30cm^3) and then extracted with diethyl ether (3 x 20cm^3). The combined organic layers were then washed successively with water (15cm^3) and brine (15cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual pale yellow oil was purified by recrystallization from hot (~85°C) toluene [filtration and washing with cold (5°C) toluene] to give the half-acid amides (221a) and (221b) as a white crystalline solid (210mg, 76%). Mp 139-141°C; TLC [methanol-dichloromethane 3:7, detection by ninhydrin spray and by short wavelength (254nm) UV lamp] showed a UV-active, non-alkaloidal compound with R_f = 0.10; δ_H (D_2O)/ppm (270MHz) 12.2 (1H, br s, RCO_2H), 11.5 (1H, s, $ArCO_2H$), 7.8 (1H, br s, NH), 8.03 (1H, d, *J* 7.3, H-6'), 7.55-7.45 (2H, m, H-3' and H-4'), 7.17 (1H, t, *J* 7.3, H-5'), 2.52-2.42 (1H, m, H-2"), 2.31-2.19 (2H, m, H_2-3"), and 1.10 (3H, br s, H_3-5"); $[\alpha]_D$ = -7.2° (c = 1.5, ethanol at 25°C) {lit. $[\alpha]_D$ = -7.0° (c = 2, ethanol at 24°C) (Goodson, 1943)}.

[See Section 2.2.10]

2.3.2.8 Preparation of Natural *S*-(-)-Methylsuccinic acid (222) (Chemical Abstracts Registry Number [2174-58-5]).

To a solution of *N*-(2-methylsuccinyl)anthranilic acids (221a) and (221b) (200mg, 0.8mmol) in diethyl ether (4cm^3) at 25°C was added aqueous hydrochloric acid solution (1M, 10cm^3) in one portion. The solution was then heated under reflux (oil-bath) and the stirring continued until completion of the reaction (24h). The mixture was cooled to 25°C (1h) and then extracted with diethyl ether (5 x 20cm^3). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to give natural *S*-(-)-methylsuccinic acid (222) as a pale yellow oil (39mg, 37%). The residual oil was purified by recrystallization from hot (~85°C) toluene [filtration and washing with cold (5°C) toluene] to give a white crystalline solid, Mp 113-115°C [lit. 115°C (Winters *et al.*, 1969)]; TLC (methanol-dichloromethane 1:4, detection by bromocresol green) $R_f = 0.4$; δ_H ($CDCl_3$)/ppm (270MHz) 10.10 (2H, br s, 2 x CO_2H), 2.97-2.89 (1H, m, 2-H), 2.72 (1H, dd, *J* 16.9 and 8.7, 3-H), 2.60 (1H, dd, *J* 16.9 and 5.5, 3-H), and 1.25 (3H, d, *J* 7.2, 5-H_3); $[\alpha]_D = -8.9°$ (c = 1.75, water at 25°C) and $[\alpha]_D = -15.1°$ (c = 2.0, ethanol at 24°C) {lit. $[\alpha]_D$ = zero° (c = 0.8, water) (Manske, 1938), $[\alpha]_D = -8.8°$ (c = 2, water at 23°C) (Goodson, 1943), and $[\alpha]_D = -15.0°$ (c = 1.9, ethanol at 24°C) (Eisenbraun and McElvain, 1955), also *R*-(+)-methylsuccinic acid $[\alpha]_D = +15.6°$ (c = 3.0, ethanol at 27°C) (Tsubokura *et al.*, 1992), $[\alpha]_D = +16.9°$ (c = 1.75, ethanol at 20°C) (Berner and Leonardsen, 1939), and $[\alpha]_D = +15.5°$ (c = 2.8, ethanol at 25°C) (Eisenbraun and McElvain, 1955)}.

[See Section 2.2.10]

2.3.2.9 Preparation of Synthetic *S*-(-)-Methylsuccinic acid (223) (Chemical Abstracts Registry Number [2174-58-5]).

Itaconic acid (224) (260mg, 2.0mmol), the procatalyst rhodium(III) chloride hydrate (8mg, 38μmol, 0.02 equiv.) and (2*S*,4*S*)-1-*tert*-butyl 4-(diphenylphosphino)-2-(diphenylphosphinomethyl)-1-pyrrolidinecarboxylate (225) (21mg, 38μmol, 0.02 equiv.) were dissolved in dimethylsulfoxide (2cm^3) at 25°C, under an atmosphere of anhydrous nitrogen. The resulting solution was stirred for 10min after which (*S*)-1-phenylethylamine (226) (484mg, 4.0mmol, 2.0 equiv.) and formic acid (0.4cm^3, 10.0mmol) were added in one portion, at 5°C (ice-bath) with stirring (an evolution of gas was noticed). The orange solution was then heated to 30°C (oil-bath) and stirring continued under an atmosphere of anhydrous nitrogen, until completion of the transfer hydrogenation reaction (17h). The mixture was cooled to 25°C (10min) and then concentrated under reduced pressure. To the resulting orange viscous oil was added aqueous hydrochloric acid solution (1M, 5cm^3) and then the acidic solution was extracted with diethyl ether (4 x 25cm^3). The combined organic layers were washed with brine (15cm^3), dried ($MgSO_4$), and concentrated under reduced pressure. The residual golden oil was purified over silica gel (17g) eluted with 10% methanol in dichloromethane plus ammonia (<1%) to give synthetic *S*-(-)-methylsuccinic acid (223) as a colourless oil (174mg, 66%). The residual oil was further purified by recrystallization from hot (~85°C) toluene [filtration and washing with cold (5°C) toluene] to give a white crystalline solid, Mp 114-116°C [lit. 115°C (Winters *et al.*, 1969)]; TLC (methanol-dichloromethane 1:4, detection by bromocresol green) $R_f = 0.4$; δ_H ($CDCl_3$)/ppm (270MHz) 10.10 (2H, br s, 2 x CO_2H), 3.00-2.87 (1H, m, 2-H), 2.73 (1H, dd, *J* 16.9 and 9.0, 3-H), 2.55 (1H, dd, *J* 16.9 and 4.8, 3-H), and 1.29 (3H, d, *J* 7.3, 5-H_3); $[\alpha]_D$ = -14.9° (c = 3.0, ethanol at 25°C) {lit. $[\alpha]_D$ = zero° (c = 0.8, water) (Manske, 1938), $[\alpha]_D$ = -8.8° (c = 2, water at 23°C) (Goodson, 1943), and $[\alpha]_D$ = -15.0° (c = 1.9, ethanol at 24°C) (Eisenbraun and McElvain, 1955), also *R*-(+)-

methylsuccinic acid $[\alpha]_D$ = +15.6° (c = 3.0, ethanol at 27°C) (Tsubokura *et al.*, 1992), $[\alpha]_D$ = +16.9° (c = 1.75, ethanol at 20°C) (Berner and Leonardsen, 1939), and $[\alpha]_D$ = +15.5° (c = 2.8, ethanol at 25°C) (Eisenbraun and McElvain, 1955)}.

[See Section 2.2.10]

2.3.2.10 Preparation of Natural *l*-Dimenthyl *S*-(-)-methylsuccinate (228) (Chemical Abstracts Registry Number [77836-38-5]).

A mixture of natural *S*-(-)-methylsuccinic acid (222) (35mg, 0.3mmol), *l*-menthol (229) (83mg, 0.5mmol, 2.0 equiv.), and a catalytic amount of *p*-toluenesulfonic acid monohydrate (5mg, 26μmol, 0.1 equiv.) in toluene ($3cm^3$) was heated under reflux (oil-bath) and stirred, under an atmosphere of nitrogen, until completion of the reaction (16h). The mixture was cooled to 25°C (1h) and then concentrated under reduced pressure. The residual amber oil was purified over silica gel (10g) eluted with 0-10% ethyl acetate in hexane to give the natural *l*-dimenthyl ester (228) as a colourless oil (91mg, 84%). TLC (ethyl acetate-hexane 1:9, detection with iodine vapour) R_f = 0.3; δ_H ($CDCl_3$)/ppm (270MHz) 4.75-4.61 (2H, m, 2 x 1'-H), 2.95-2.82 (1H, m, 2-H), 2.77-2.68 (1H, m, 3-H), 2.39-2.31 (1H, m, 3-H), 2.03-1.93 (2H, m, 2'/4'/5'-H_2), 1.90-1.80 (2H, m, 2'/4'/5'-H_2), 1.72-1.62 (4H, m, 2 x 2'/4'/5'-H_2), 1.56-1.30 (4H, m, 2 x 2'/4'/5'-H_2), 1.21 (3H, d, *J* 7.2, 5-H_3), 1.10-0.78 (18H, m, 4 x 9'-H_3, 2 x 3'-, 6'-, and 8'-H), 0.77 (3H, d, *J* 7.0, 7'-H_3), and 0.75 (3H, d, *J* 7.0, 7'-H_3); δ_C ($CDCl_3$)/ppm (100.4MHz) (Tsubokura *et al.*, 1992) 174.8 (C-1), 171.4 (C-4), 74.3 (C-1'), 74.2 (C-1'), 47.0 (C-6'), 46.9 (C-6'), 40.9 (C-2'), 40.8 (C-2'), 37.9 (C-3), 36.1 (C-2), 34.3 (C-4'/5'), 34.2 (C-4'/5'), 31.4 (C-8'), 26.2 (C-8'), 26.1 (C-3'), 26.0 (C-3'), 23.4 (C-4'/5'), 23.3 (C-4'/5'), 22.0 (br, C-9'), 20.8 (C-9'), 20.7 (C-9'), 17.0 (C-5), 16.3 (C-7'), and 16.0 (C-7'); $C_{25}H_{44}O_4$ requires MW 408. Found: m/z (EI) 408 (M^+, 100%).

[See Section 2.2.10]

2.3.2.11 Preparation of Synthetic *l*-Dimenthyl *S*-(-)-methylsuccinate (230) (Chemical Abstracts Registry Number [77836-38-5]).

A mixture of synthetic *S*-(-)-methylsuccinic acid (223) (135mg, 1.0mmol), *l*-menthol (229) (320mg, 2.0mmol, 2.0 equiv.), and a catalytic amount of *p*-toluenesulfonic acid (19mg, 0.1mmol, 0.1 equiv.) in toluene (10cm^3) was heated under reflux (oil-bath) and stirred, under an atmosphere of nitrogen, until completion of the reaction (17h). The mixture was cooled to 25°C (1h) and then concentrated under reduced pressure. The residual amber oil was purified over silica gel (40g) eluted with 0-10% ethyl acetate in hexane to give the synthetic *l*-dimenthyl ester (230) as a colourless oil (330mg, 79%). TLC (ethyl acetate-hexane 1:9, detection with iodine vapour) $R_f = 0.3$; δ_H ($CDCl_3$)/ppm (270MHz) 4.74-4.63 (2H, m, 2 x 1'-H), 2.91-2.83 (1H, m, 2-H), 2.78-2.67 (1H, m, 3-H), 2.39-2.30 (1H, m, 3-H), 2.03-1.96 (2H, m, 2'/4'/5'-H_2), 1.89-1.83 (2H, m, 2'/4'/5'-H_2), 1.70-1.64 (4H, m, 2 x 2'/4'/5'-H_2), 1.41-1.26 (4H, m, 2 x 2'/4'/5'-H_2), 1.21 (3H, d, *J* 7.2, 5-H_3), 1.12-0.86 (18H, m, 4 x 9'-H_3, 2 x 3'-, 6'-, and 8'-H), 0.75 (3H, d, *J* 7.2, 7'-H_3), and 0.75 (3H, d, *J* 7.0, 7'-H_3); δ_C ($CDCl_3$)/ppm (67.8MHz) (Tsubokura *et al.*, 1992) 174.8 (C-1), 171.4 (C-4), 74.3 (C-1'), 74.2 (C-1'), 47.0 (C-6'), 46.9 (C-6'), 40.9 (C-2'), 40.8 (C-2'), 37.9 (C-3), 36.1 (C-2), 34.3 (C-4'/5'), 34.2 (C-4'/5'), 31.4 (C-8'), 26.2 (C-8'), 26.1 (C-3'), 26.0 (C-3'), 23.4 (C-4'/5'), 23.3 (C-4'/5'), 22.0 (br, C-9'), 20.8 (C-9'), 20.7 (C-9'), 17.0 (C-5), 16.3 (C-7'), and 16.0 (C-7'); $C_{25}H_{44}O_4$ requires MW 408. Found: m/z (EI) 408 (M^+, 100%).

[See Section 2.2.10]

2.3.2.12 Preparation of *l*-Dimenthyl (*RS*)-methylsuccinate (231)

A mixture of (*RS*)-methylsuccinic acid (232) (200mg, 1.5mmol), *l*-menthol (229) (470mg, 3.0mmol, 2.0 equiv.), and a catalytic amount of *p*-toluenesulfonic acid (29mg, 0.15mmol, 0.1 equiv.) in toluene (15cm^3) was heated under reflux (oil-bath) and stirred, under an atmosphere of nitrogen, until completion of the

reaction (14h). The mixture was cooled to 25°C (20min) and then concentrated under reduced pressure. The residual amber oil was purified over silica gel (60g) eluted with 0-10% ethyl acetate in hexane to give the *l*-dimenthyl ester (231) as a colourless oil (545mg, 89%). TLC (ethyl acetate-hexane 1:9, detection with iodine vapour) $R_f = 0.3$; δ_H ($CDCl_3$)/ppm (270MHz) 4.74-4.62 (2H, m, 2 x 1'-H), 2.93-2.83 (1H, m, 2-H), 2.77-2.68 (1H, m, 3-H), 2.39-2.30 (1H, m, 3-H), 2.03-1.93 (2H, m, 2'/4'/5'-H_2), 1.90-1.80 (2H, m, 2'/4'/5'-H_2), 1.71-1.63 (4H, m, 2 x 2'/4'/5'-H_2), 1.53-1.31 (4H, m, 2 x 2'/4'/5'-H_2), 1.21 (3H, 2 x d, 2 x *J* 7.0, 5-H_3), 1.15-0.79 (18H, m, 4 x 9'-H_3, 2 x 3'-, 6'-, and 8'-H), 0.76 (3H, d, *J* 7.0, 7'-H_3), and 0.74 (3H, d, *J* 6.8, 7'-H_3); δ_C ($CDCl_3$)/ppm (100.4MHz) (Tsubokura *et al.*, 1992) 174.8 (C-1), 174.6 (C-1), 171.4 (C-4), 171.2 (C-4), 74.3 (C-1'), 74.2 (C-1'), 47.0 (C-6'), 46.9 (C-6'), 40.9 (C-2'), 40.8 (C-2'), 38.0 (C-3), 37.9 (C-3), 36.1 (br, C-2), 34.3 (C-4'/5'), 34.2 (C-4'/5'), 31.4 (C-8'), 26.2 (C-8'), 26.1 (C-3'), 26.0 (C-3'), 23.4 (C-4'/5'), 23.3 (C-4'/5'), 22.0 (br, C-9'), 20.8 (C-9'), 20.7 (C-9'), 17.2 (C-5), 17.0 (C-5), 16.3 (C-7'), and 16.0 (C-7'); $C_{25}H_{44}O_4$ requires MW 408. Found: m/z (EI) 408 (M^+, 100%).

[See Section 2.2.10]

2.3.2.13 Preparation of Synthetic *S*-(-)-Methylsuccinic anhydride (233) (Chemical Abstracts Registry Number [6973-20-2]).

A suspension of synthetic *S*-(-)-methylsuccinic acid (223) (170mg, 1.3mmol) in acetyl chloride (1.5cm^3) was stirred at 25°C until completion of the reaction (15h). The resulting solution was concentrated under reduced pressure and the residual colourless oil was recrystallized from hot (~65°C) toluene-hexane (1:1) [filtration and washing with cold (5°C) toluene] to give synthetic *S*-(-)-methylsuccinic anhydride (233) as a white crystalline solid (101mg, 69%). Mp 69-70.5°C [lit. Mp 69.5°C (Berner and Leonardsen, 1939)]; TLC (methanol-dichloromethane 1:9, detection with iodine vapour) $R_f = 0.3$; δ_H ($CDCl_3$)/ppm (270MHz) 3.22-3.09 (2H, m, 2- and 3-H], 2.61 (1H, dd, *J* 11.9 and 10.6, 3-H), and 1.43 (3H, d, *J* 7.0, 5-H_3); $[\alpha]_D = -36.3°$ (c = 3.5, dioxane at 23°C) {lit. *R*-

(+)-methylsuccinic anhydride $[\alpha]_D$ = +32.6° (c = 15.0, dioxane at 20°C) (Berner and Leonardsen, 1939)}.

[See Sections 2.2.10 and 2.2.11]

2.3.2.14 Preparation of Inuline (40)
(Chemical Abstracts Registry Number [22413-78-1]).

To a stirred solution of isatoic anhydride (248) (114mg, 0.7mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (8mg, 0.1mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (3cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added lycoctonine (2) (360mg, 0.8mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (16h). The mixture was cooled to 25°C (1h) and then partitioned between ethyl acetate (3cm^3) and water (3cm^3). The aqueous layer was extracted with ethyl acetate (3 x 5cm^3) and the combined organic layers were then washed successively with water (3 x 5cm^3) and brine (3cm^3), dried ($MgSO_4$), and concentrated under reduced pressure. The residual amber oil was purified over silica gel (10g) eluted with 0-10% methanol in dichloromethane to give inuline (40) as a colourless oil (90mg, 22%). TLC (dichloromethane-methanol-ammonia 100:10:1, detection by Dragendorff Munier spray) R_f = 0.7; δ_H ($CDCl_3$)/ppm (400MHz) (Pelletier *et al.*, 1984) 7.79 (1H, dd, *J* 8.4 and 1.6, H-6'), 7.27 (1H, ddd, *J* 8.1, 7.4, and 1.6, H-4'), 6.70-6.64 (2H, m, H-3' and H-5'), 5.77 (2H, br s, NH_2), 4.18-4.01 (2H, m, H-β-18 and H-α-18), 3.93 (1H, s, H-6), 3.61 (1H, dd, *J* 4.6 and 4.6, H-14), 3.41 [3H, s, C(6)OC*H*$_3$], 3.38 [3H, s, C(16)OC*H*$_3$], 3.35 [3H, s, C(14)OC*H*$_3$], 3.27 [3H, s, C(1)OC*H*$_3$], 3.24-3.19 (1H, m, H-16), 3.08 (1H, dd, *J* 4.6 and 6.7, H-9), 3.00 (1H, dd, *J* 7.0 and 10.1, H-β-1), 2.96-2.89 (2H, m, H-17 and 1 of NC*H*$_2$ CH_3), 2.87-2.77 (1H, m, 1 of NC*H*$_2$ CH_3), 2.73 (1H, d, *J* 11.6, H-α-19), 2.61 (1H, dd, *J* 8.9 and 15.3, H-α-15), 2.50-2.42 (2H, m, H-α-12 and H-β-19), 2.34 (1H, dd, *J* 4.6 and 7.0, H-13), 2.25-2.03 (2H, m, H-β-2 and H-α-2), 2.00-1.93 (1H, m, H-10), 1.89-1.80 (1H, m, H-β-12), 1.79-1.74 [2H, m, H-α-3 and C(8)OH], 1.72-1.65 [3H,

m, H-5, H-β-15, and C(7)OH], 1.63-1.52 (1H, m, H-β-3), and 1.07 (3H, t, *J* 7.0, NCH_2CH_3); δ_C ($CDCl_3$)/ppm (67.8MHz) (Pelletier *et al.*, 1984) 167.9 (CO), 150.9 (C-2'), 134.4 (C-4'), 130.8 (C-6'), 117.0 (C-3'), 116.3 (C-5'), 110.4 (C-1'), 91.0 (C-6), 88.6 (C-7), 84.1 (C-1), 84.0 (C-14), 82.7 (C-16), 77.6 (C-8), 68.7 (C-18), 64.6 (C-17), 58.0 [C(6)OCH_3], 57.9 [C(14)OCH_3], 56.3 [C(16)OCH_3], 55.9 [C(1)OCH_3], 52.6 (C-19), 51.1 (NCH_2CH_3), 50.4 (C-5), 49.1 (C-11), 46.2 (C-10), 43.3 (C-9), 38.3 (C-13), 37.7 (C-4), 33.7 (C-15), 32.3 (C-3), 28.8 (C-12), 26.2 (C-2), and 14.1 (NCH_2CH_3); $C_{32}H_{46}N_2O_8$ requires MW 586 and C, 65.5; H, 7.9; N, 4.8%; Found: m/z (+) FAB 587 (MH^+, 100%), 571 [$(M-CH_3)^+$, 8%], 120 ($C_7H_6NO^+$, 15%), (-) FAB 585 (M^--1, 44%) and C, 65.6; H, 7.8; N, 4.7%; $[\alpha]_D$ = +49.7° (c = 0.75, ethanol at 25°C), {lit. $[\alpha]_D$ = +50.2° (c = 1.1, ethanol at 24°C) (Pelletier *et al.*, 1981b)}.

[See Sections 2.2.7, 2.2.8, 2.2.9 and 2.2.11]

2.3.2.15 Preparation of Semi-Synthetic MLA (250)

A solution of synthetic *S*-(-)-methylsuccinic anhydride (233) (22mg, 0.2mmol, 1.25 equiv.) and inuline (40) (90mg, 0.2mmol) in anhydrous dichloromethane (5cm^3) was stirred at 25°C, under an atmosphere of nitrogen (40h). To the resulting yellow solution was added 1,1'-carbonyldiimidazole (251) (124mg, 0.8mmol, 5.00 equiv.) and stirring continued at 25°C, under an atmosphere of nitrogen, until completion of the reaction (40h). The solution was concentrated under reduced pressure and the residual yellow foam was purified over silica gel (6g) eluted with 0-10% methanol in dichloromethane to give semi-synthetic MLA (250) as a colourless oil (60mg, 57%). TLC (methanol-dichloromethane 1:9, detection by Dragendorff Munier spray) R_f = 0.5; δ_H ($CDCl_3$)/ppm (270MHz) (Chen and Wu, 1990 and Sun and Benn, 1992) 8.05 (1H, d, *J* 7.6, H-6'), 7.70 (1H, t, *J* 7.6, H-4'), 7.55 (1H, t, *J* 7.6, H-5'), 7.30 (1H, d, *J* 7.6, H-3'), 4.15-4.00 (2H, m, H-β-18 and H-α-18), 3.85 (1H, s, H-6), 3.60 (1H, dd, *J* 4.8 and 4.6, H-14), 3.45 [3H, s, C(6)OCH_3], 3.42 [3H, s, C(14)OCH_3], 3.38 [3H, s, C(16)OCH_3], 3.28 [3H, s, C(1)OCH_3], 3.24-3.17 (1H, m, H-16), 3.10-2.97 (3H,

m, H-9, H-3", and H-2"), 2.95-2.92 (3H, m, H-β-1, H-17, and 1 of NCH_2 CH_3), 2.73-2.69 (2H, m, H-α-19 and 1 of NCH_2 CH_3), 2.64-2.37 (4H, m, H-α-15, H-3", H-α-12, and H-β-19), 2.35-2.31 (1H, m, H-13), 2.20-2.15 (1H, m, H-α-2), 2.12-2.00 (1H, m, H-β-2), 1.97-1.54 (6H, m, H-10, H-β-12, H-α-3, H-5, H-β-15, and H-β-3), 1.49-1.42 (3H, m, H_3-5"), and 1.07 (3H, t, *J* 7.0, NCH_2CH_3); δ_C ($CDCl_3$)/ppm (90.6MHz) (Chen and Wu, 1990 and Sun and Benn, 1992) 179.8 (C-1"), 175.8 (C-4"), 164.1 (CO), 133.7 (C-4'), 133.1 (C-2'), 131.0 (C-6'), 130.0 (C-3'), 129.4 (C-5'), 126.9 (C-1'), 90.7 (C-6), 88.5 (C-7), 83.9 (C-1), 83.0 (C-14), 82.5 (C-16), 77.6 (C-8), 69.5 (C-18), 64.5 (C-17), 58.1 [C(6)OCH_3], 57.8 [C(14)OCH_3], 56.3 [C(16)OCH_3], 55.8 [C(1)OCH_3], 52.4 (C-19), 50.9 (NCH_2CH_3), 50.4 (C-5), 49.1 (C-11), 46.2 (C-10), 43.2 (C-9), 38.1 (C-13), 37.6 (C-4), 37.0 (C-3"), 35.2 (C-2"), 33.6 (C-15), 32.0 (C-3), 28.7 (C-12), 26.1 (C-2), 16.4 (C-5"), and 14.1 (NCH_2CH_3); $C_{37}H_{50}N_2O_{10}$ requires MW 682 and C, 65.1; H, 7.4; N, 4.1%; Found: m/z (+) FAB 683 (MH^+, 100%), 668 [$(MH-CH_3)^+$, 7%], 652 [$(MH-OCH_3)^+$, 5%], 216 ($C_{12}H_{10}NO_3^+$, 16%), (-) FAB 681 (M^--1, 100%) and C, 65.0; H, 7.5; N, 4.2%. [See Section 2.2.11]

CHAPTER 3

LC STUDIES

3.1 AIMS

The aims of these chromatographic investigations were:

i) to develop a sensitive assay for MLA (1) to enable the routine monitoring of MLA levels in other naturally occurring alkaloid samples and their derivatives prior to biological testing.

ii) to develop a reverse-phase HPLC system for the analysis of norditerpenoid alkaloids which utilizes a volatile mobile phase, providing the flexibility for routine analytical use and adaptability to LC/MS applications (See Sections 3.2.8 and 3.3.6) and scale-up to semi-preparative alkaloid isolation.

iii) to optimize the sampling method, the calibration technique, the column resolution, and the detection (Hamilton and Sewell, 1986) of the standard methodology to be used for the assessment of the chemical purity of the plant extracts and semi-synthetic alkaloids, and thus obtain precise, accurate, sensitive, and reproducible quantitative results.

3.2 RESULTS AND DISCUSSION

3.2.1 Background

In recent years, much research has been directed towards the development of methods for identification and quantitation of alkaloids due to the great interest in tracing toxic alkaloids in food or fodder and because many drugs of abuse are alkaloids (Verpoorte and Niessen, 1994). A number of procedures have been developed for the analysis of the closely related *Aconitum* alkaloids, including paper electrophoresis, TLC, multi-buffered paper partition chromatography, and (capillary) gas-liquid chromatography (Hikino *et al.*, 1983 and Manners and Ralphs, 1989). However, HPLC has proved to be a more precise and less time-consuming technique for the aconitine alkaloids (Hikino *et al.*, 1983) as well as for many other types of alkaloids (for example, strychnine, nicotine, indole alkaloids, ergot alkaloids, and tropane alkaloids) (Verpoorte and Niessen, 1994). It is surprising therefore that despite the extensive phytochemical literature on *Delphinium* alkaloids, there are relatively few reports concerning the application of HPLC to this group.

One problem has been the detection of those alkaloids which lack an aromatic chromophore. Majak and co-workers (Majak *et al.*, 1987, Majak and Engelsjord, 1988, and Majak, 1993) reported a reverse-phase, ion-pair HPLC system for alkaloids such as MLA (1) possessing a suitable chromophore (UV detection at 277nm). However, the external standard method of quantitation they used would be insufficiently precise for our purposes and their use of sodium hexanesulfonate precluded LC/MS and scale-up for semi-preparative use.

Manners and Pfister (1993) reported a normal-phase analytical HPLC method for the quantification of *Delphinium* alkaloids. However, their system (using

hexane-95% aqueous isopropyl alcohol as mobile phase) did not fully resolve MLA (1) from lycoctonine (2), as revealed by UV detection at 220nm.

Several different research groups have reported HPLC methods for *Aconitum* alkaloids (Hikino *et al.*, 1981 and 1983, Kulanthaivel and Pelletier, 1987 and, Niitsu *et al.*, 1990). Many of the methods used a large organic anionic counter-ion in the mobile phase and were therefore deemed unsuitable for scale-up to a semi-preparative level or LC/MS. In addition, a number of the methods used mobile phases containing tetrahydrofuran, which can cause corrosion of seals in HPLC pumps and is for that reason not desirable.

Hikino and co-workers described a reverse-phase quantitative assay for *Aconitum* alkaloids (Hikino *et al.*, 1981 and 1983), using a chemically-bonded octadecylsilane (ODS) column and a 0.05M phosphate buffer (pH 2.7)-tetrahydrofuran (89:11) mobile phase. Hikino *et al.* (1981 and 1983) developed a similar alternative method involving ion-pair chromatography (0.01M sodium hexanesulfonate) giving sharp symmetrical peaks and detection limits for the monitored aconitine type alkaloids of 12ng and 15ng with UV detection at 254nm. The former method was found by Kulanthaivel and Pelletier (1987) to suffer from a lack of resolution and tailing of peaks. The qualitative reverse-phase HPLC system reported by Kulanthaivel and Pelletier (1987) used a simple mobile phase consisting of ammonium carbonate and tetrahydrofuran. In 1990, Niitsu *et al.* reported a normal-phase preparative HPLC system for *Aconitum* alkaloids using a simple mobile phase consisting of cyclohexane-ethyl acetate-diethylamine. Wada *et al.* (1993) developed a LC/MS system for *Aconitum* alkaloids using 0.05M ammonium acetate-acetonitrile-tetrahydrofuran (60:25:15). The detection limits of the test alkaloids were 1-5ng per injection, but they were poorly resolved chromatographically. Yunusov (1993) reviewed various HPLC methods for *Aconitum* alkaloids published in the literature between the end of 1989 and the beginning of 1992, including a system using a ZORBAX column and a methanol-water-chloroform-

triethylamine (70:30:1:0.1) mobile phase and a method to separate and quantify lappaconitine (39) and its metabolites in rat urine, using an ODS chemically-bonded column and an ammonium acetate-methanol-acetonitrile mobile phase.

3.2.2 Choice of Stationary Phase

A 25cm x 4.6mm i. d. Hypersil ODS 5μm column was chosen for our investigations because the specifications for such a column were expected to give a high separation efficiency (Hamilton and Sewell, 1986). Chemically-bonded ODS is a widely used stationary phase (Hamilton and Sewell, 1986). The non-polar nature of the C_{18} alkyl side-chain in the Si-O-$SiC_{17}H_{34}CH_3$ group formed means that the stationary phase is less polar than the mobile phase and it is therefore defined as reverse-phase mode (Hamilton and Sewell, 1986). The Hypersil ODS column stationary phase is described as monomeric with 9% surface coverage. It is the bonded phase coverage which determines the number of surface silanols available and the type of reaction. Residual silanol groups are capped by treatment with trimethylchlorosilane, thus reducing the chance of tailing (Hamilton and Sewell, 1986).

3.2.3 Choice of Detector

UV absorption is the most widely used method of detection in HPLC, being sensitive, reproducible, and easy to operate. With sensitivities of 0.001 absorbance unit, full scale deflection, and noise levels typically 1%, it is possible possible to detect as little as 1ng of solute with a moderate UV absorbance (Hamilton and Sewell, 1986). The wide linear dynamic range (10^4) of these detectors make it possible to measure both trace and major components on the same chromatogram. Ideally the detector should have a usable sensitivity of better than 0.1μg of sample in $1cm^3$ of mobile phase. The approximate sensitivity for suitable trace solutes is $0.0005\mu g.cm^{-3}$ for UV detectors (Hamilton and Sewell, 1986). MLA (1) exhibits a UV absorption

maximum at 271nm (in addition to a λ_{max} at 229nm) so the assay was carried out near this value, at 270nm. The UV data was comparable to that of Cookson *et al.* (1954), who saw a maximum at 273nm (as well as λ_{max} = 230nm) with a molar extinction coefficient ε 3,500 (and ε 15,300).

3.2.4 Choice of Mobile Phase

When using a chemically-bonded silica gel column in the analysis of basic substances, careful choice of mobile phase is necessary to avoid tailing peaks and rapid elution of polar solutes, such as ions. Poor shaped peaks are prevalent when using methanol and water due to interaction with the free silanol groups on the stationary phase surface (Hamilton and Sewell, 1986). There are two ways around the problem of a solute eluting too quickly, ion-pairing and ion-suppression (Hamilton and Sewell, 1986). The former involves the use of a large organic anion in the mobile phase in order to form a relatively non-polar ion-pair with the cationic solute present. In this case, in order to ensure the alkaloid is fully ionized and thus giving the maximum concentration of the ionic form, it is necessary to use a low pH. Alternatively, by making the mobile phase less acidic (by buffering at relatively high pH values), the ionization of the alkaloid can be suppressed, thus making it less polar and more retained by the column by depending on the lipophilic character of the alkaloid (rather than the ion-pair formed). If the pK_a of the alkaloidal solute is approximately 8, then based on the fact that ion-suppression only operates within approximately 2-3 pH units of the pK_a, ion-suppression will start to have an effect at pH 5-6. Therefore, our alkaloids, which are relatively weak bases, are suitable candidates for the development of a system using ion-suppression, in view of the fact that it is considered unwise to use a higher pH than 7 with silica-based phases.

Chromatographic behaviour is not only dependent on the pH of the mobile phase, but on the nature of the organic solvent and the overall composition (Hamilton and Sewell, 1986). The property of a solvent responsible for the solute-solvent interactions that produces solute elution from the column, that the chemist thinks of as polarity, encompasses both hydrogen bonding and strong dipole interactions. There are many tables of solvent properties (for example, eluant strength function, solubility parameter, polarity index) and of classification of solvents available to aid in the selection of the mobile phase (Hamilton and Sewell, 1986). However, the first consideration, in this selection of an appropriate mobile phase, is that the solvent must be capable of dissolving the solutes. For reverse-phase ion-suppression conditions desired for our system, water mixed with methanol, acetonitrile, dioxane or tetrahydrofuran is the most popular solvent (Hamilton and Sewell, 1986). Acetonitrile was chosen as the organic component as it has low viscosity, favourable vapour pressure, and UV transparency (Hamilton and Sewell, 1986). Ammonium acetate (Mp 112-114°C) and formic acid (Bp 101°C) were chosen to make up the aqueous buffer component because of the volatility of these species relative to other commonly used inorganic buffer components. The optimization experiments carried out, examined the effects of acetonitrile concentration and pH of the aqueous component of the eluant on the capacity factor values (κ') of MLA (1) and lappaconitine (39).

Where $\kappa' = \frac{t_R - t_O}{t_O}$ Equation I

and t_R = retention time

t_O = the time taken for the solvent molecule (or an unretained compound) to traverse the column.

3.2.4.1 Theory for Optimization of the Mobile Phase in HPLC

The quality of an HPLC analysis is assessed by measurement of the resolution between two adjacent peaks. Resolution (R_S), a measure of the separation achieved, has three contributory factors (Hamilton and Sewell, 1986): column efficiency (N), solute retention (κ'), and selectivity (α).

[See Section 3.3.2]

$$R_S = \frac{1}{4} \times \sqrt{N_{MLA}} \times \frac{\kappa'_{MLA}}{(1 + \kappa'_{MLA})} \times \frac{(\alpha - 1)}{\alpha} \qquad \text{Equation II}$$

where $$N_{MLA} = \frac{5.54 \times t_{RMLA}^2}{w_{1/2MLA}^2} \qquad \text{Equation III}$$

$$\alpha = \frac{\kappa'_{MLA}}{\kappa'_{Lapp}} \qquad \text{Equation IV}$$

where $w_{1/2}$ = peak width at half peak height

or $$R_S = \frac{t_{RMLA} - t_{RLapp}}{\frac{1}{2}(w_{MLA} + w_{Lapp})} \qquad \text{Equation V}$$

where w = peak width at the baseline

Theoretical and experimental values for R_S can be obtained using Equations II and V, respectively. An $R_S = 1.5$ value for symmetrical peaks is taken to represent baseline separation. An $R_S = 0.8$ value, equivalent to 98% separation of two components, is normally considered the lowest practical value for qualitative analysis (Hamilton and Sewell, 1986).

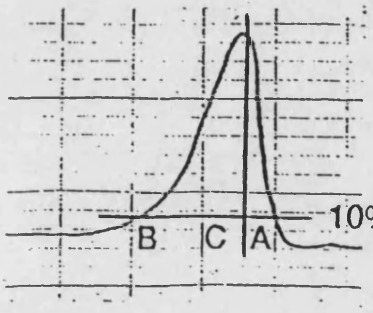
The column efficiency (N) is mainly a function of the column and the way it is packed (Hamilton and Sewell, 1986). It is expressed by the determination of the number of theoretical plates N where the larger the number of theoretical plates the more likely the column is to carry out the expected separation. An important factor for trace analysis, N of at least 2000-4000 is a prerequisite (Hamilton and Sewell, 1986).

Column capacity ratio (κ') is used to measure the retention of an analyte in HPLC systems. It is dependent on solvent strength (polarity, pH, or ionic strength) and solvent composition as discussed above and it is important that κ' is in the range 1 to 10 (Hamilton and Sewell, 1986).

The selectivity factor or relative retention (α) is a measure of the ability of the chromatographic system to recognize chemical differences between the two components. Changes in the mobile phase composition rather than the solvent strength are important in maximizing α (Hamilton and Sewell, 1986). It takes into consideration only retention times and not peak width. Values of α must be greater than 1.05 and values greater than 1.2 are desirable (Hamilton and Sewell, 1986).

A peak asymmetry factor (A_S) = 0.9-1.2 represents a good column and is obtained for symmetrical Gaussian peaks (Hamilton and Sewell, 1986).

Where $A_S = \frac{CB}{AC}$ See diagram Equation VI



A skewed peak (fronting $A_S \leq 0.9$ or tailing $A_S \geq 1.2$) is the result of a non-linear relationship between the concentration of the sample molecules in the stationary and mobile phases, such that the retention time will vary with sample size. It is important to show that peaks are symmetrical, especially when peak height measurements are to used in the construction of a calibration curve (Hamilton and Sewell, 1986).

3.2.4.2 Variation in Organic Modifier Content

The first stage in the optimization of the mobile phase was to investigate the effect of the ratio of ammonium acetate buffer and acetonitrile in the mobile phase [Figures (26) and (27)]. Figure (28) shows that linear plots were obtained for the logarithm of κ' over the concentration range of 25-40% acetonitrile.

After examining the chromatograms, it was decided that the most satisfactory results were obtained using the mobile phase containing 30% acetonitrile, based on peak shape, retention times, and resolution.

[See Section 3.3.2.1]

3.2.4.3 pH Profile

The effect of the pH of the ammonium acetate buffer on κ' is shown in Figure (31). [See Figures (29) and (30)]. Linear plots of κ' were obtained for MLA (1) and lappaconitine (39) over the pH range of 3-5.

After examining the chromatograms, it was concluded that the most satisfactory results were obtained using the mobile phase buffered to pH 5, based on peak symmetry, convenient retention times, and resolution.

[See Section 3.3.2.2]

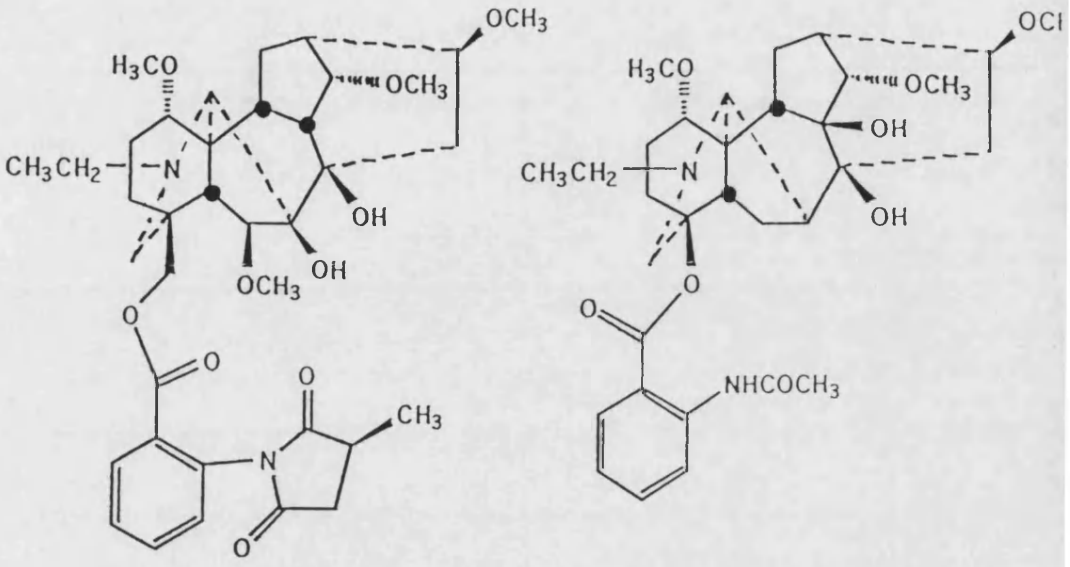


Figure (26) Separation of MLA (1) (10μg.cm^{-3}) and lappaconitine (39) (25μg.cm^{-3}) with 0.2M aqueous ammonium acetate solution (pH 4.0)-acetonitrile (75:25 V/v).

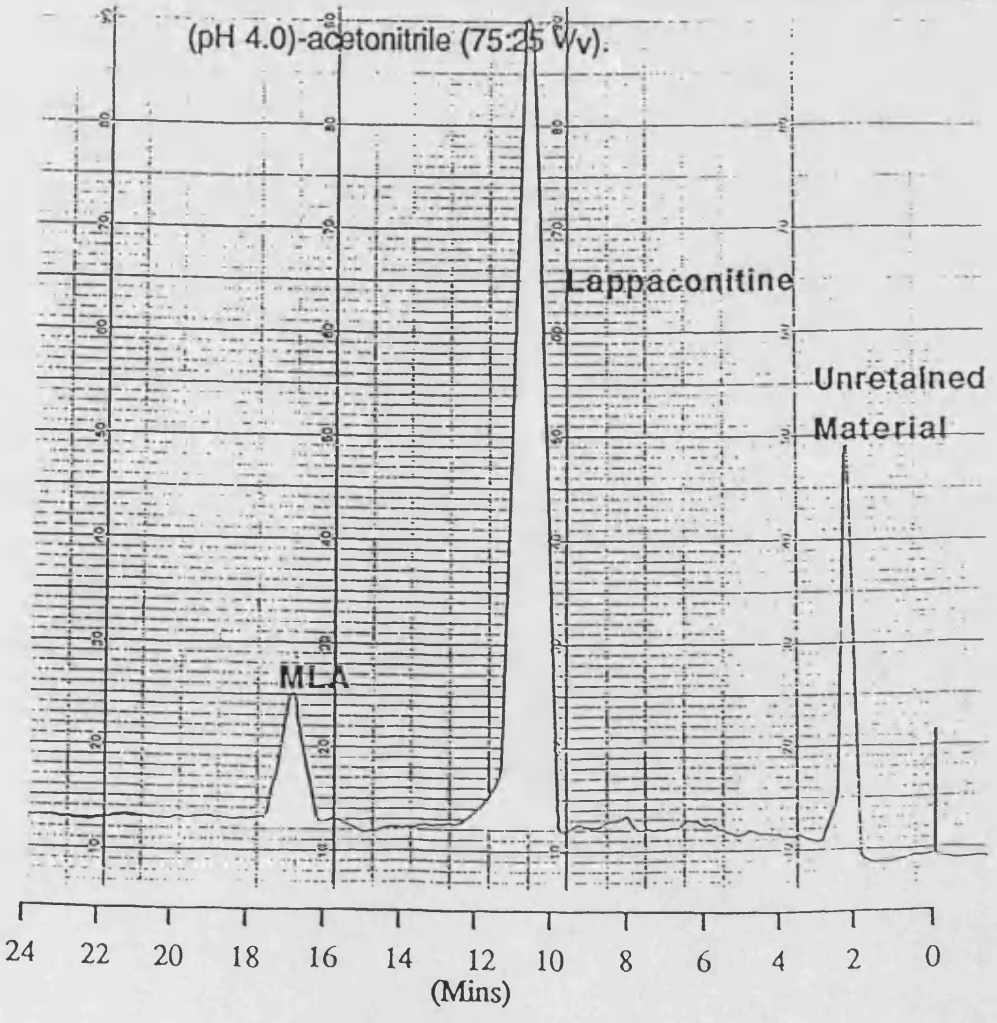


Figure (27) Separation of MLA (1) (10μg.cm^{-3}) and lappaconitine (39) (25μg.cm^{-3}) with 0.2M aqueous ammonium acetate solution (pH 4.0)-acetonitrile (60:40 v/v).

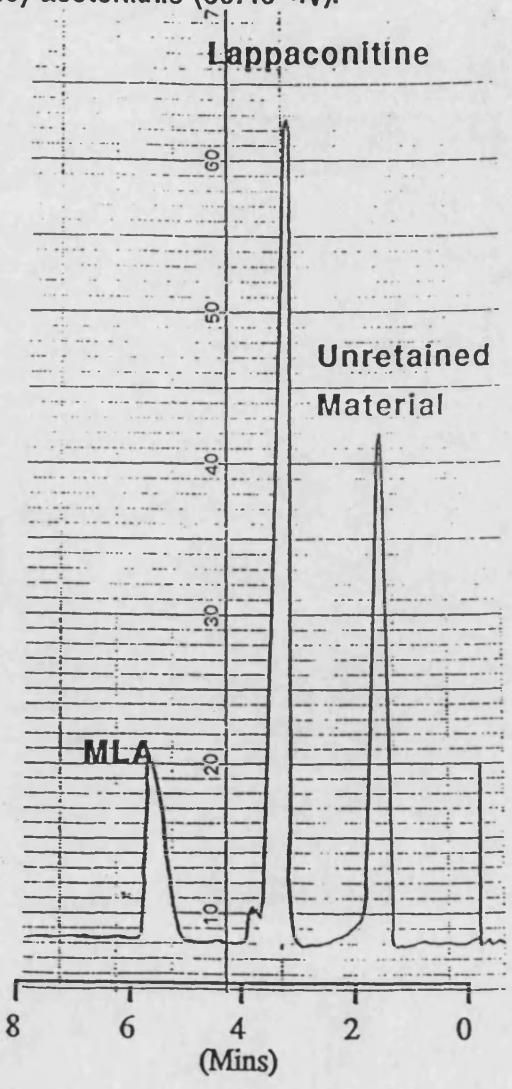


Figure (28) Effect of Variation of Acetonitrile Content on log κ'
(with 0.2M aqueous ammonium acetate solution at constant pH 4.0).

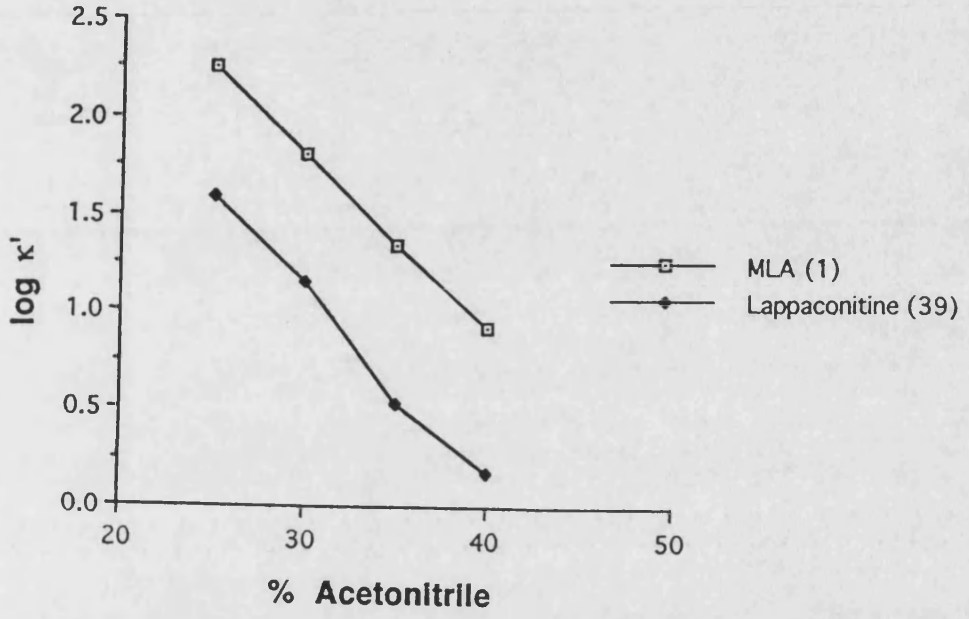


Figure (29) Separation of MLA (1) (10μg.cm^{-3}) and lappaconitine (39) (25μg.cm^{-3}) with 0.2M aqueous ammonium acetate solution (pH 3.0)-acetonitrile (65:35 v/v).

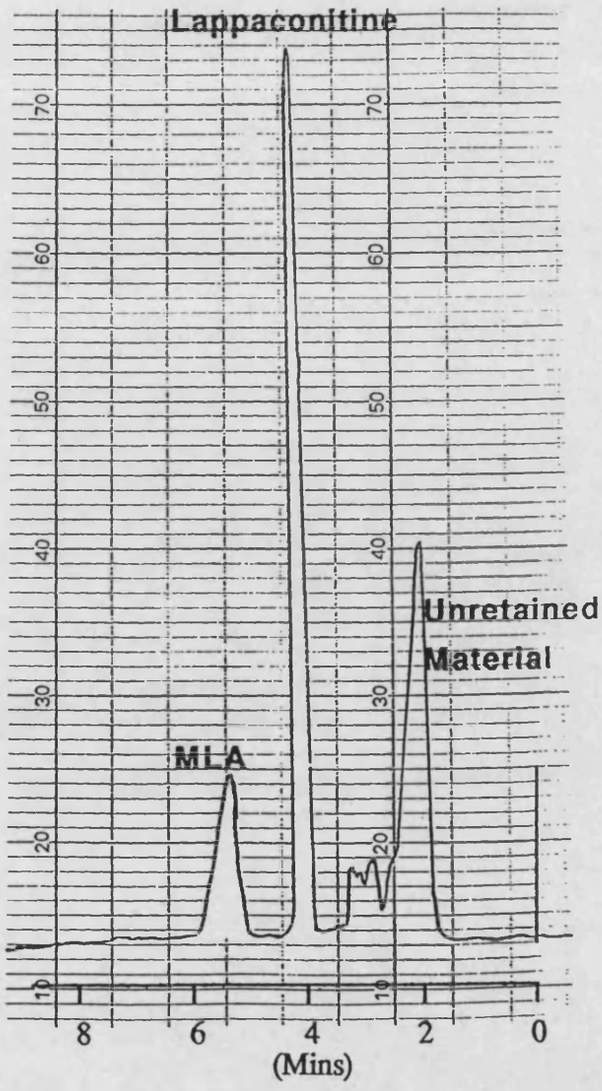


Figure (30) Separation of MLA (1) (10μg.cm^{-3}) and lappaconitine (39) (25μg.cm^{-3}) with 0.2M aqueous ammonium acetate solution (pH 5.0)-acetonitrile (65:35 v/v).

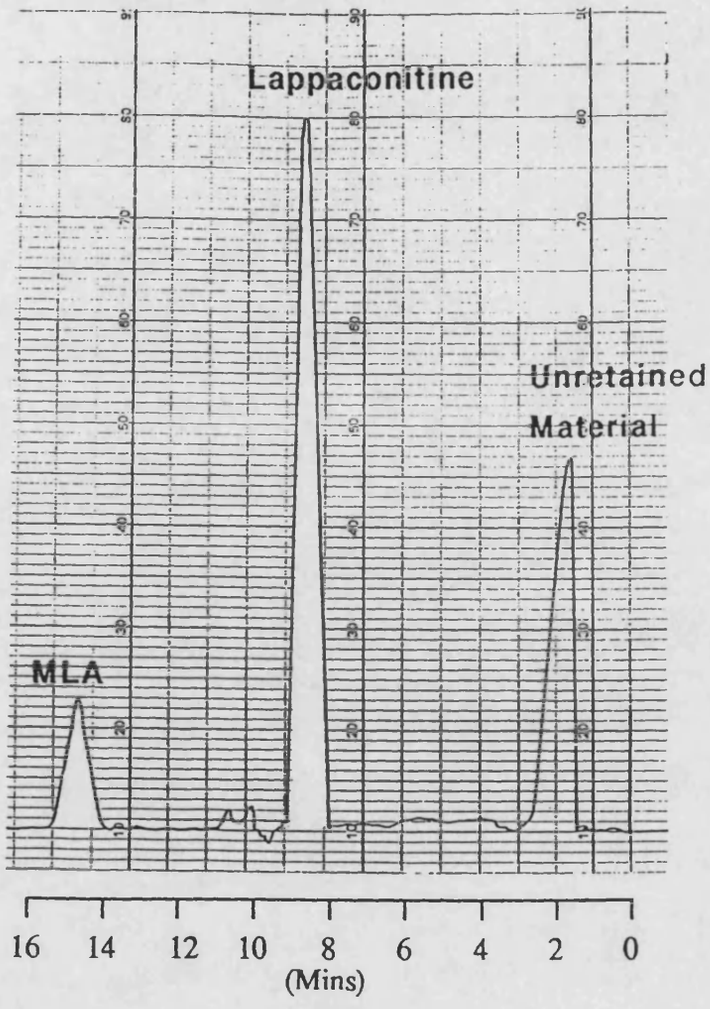
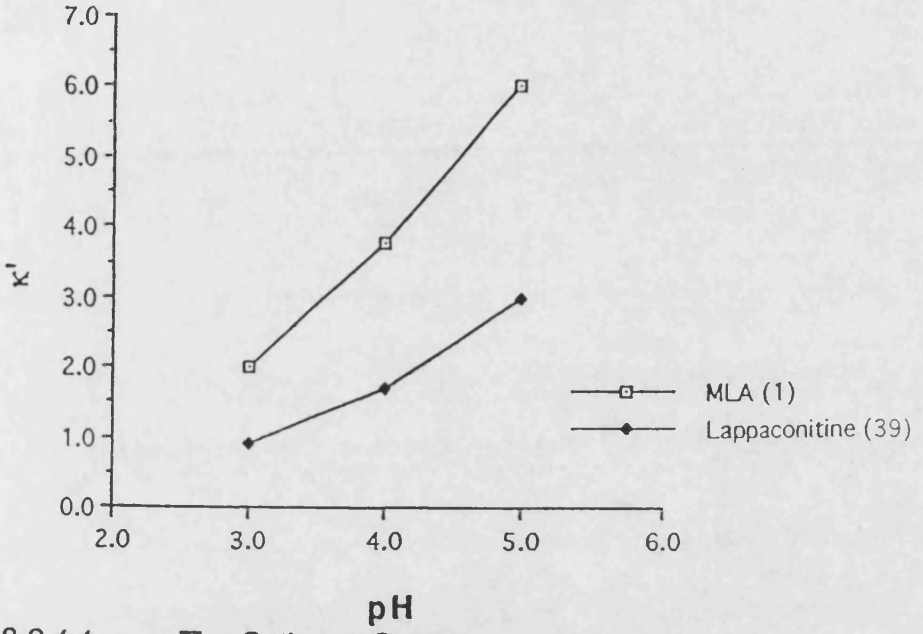


Figure (31) Effect of Variation of pH of the aqueous component of the mobile phase (0.2M aqueous ammonium acetate solution) on κ' [at constant acetonitrile content of 35% (v/v)].



3.2.4.4 The Optimum System

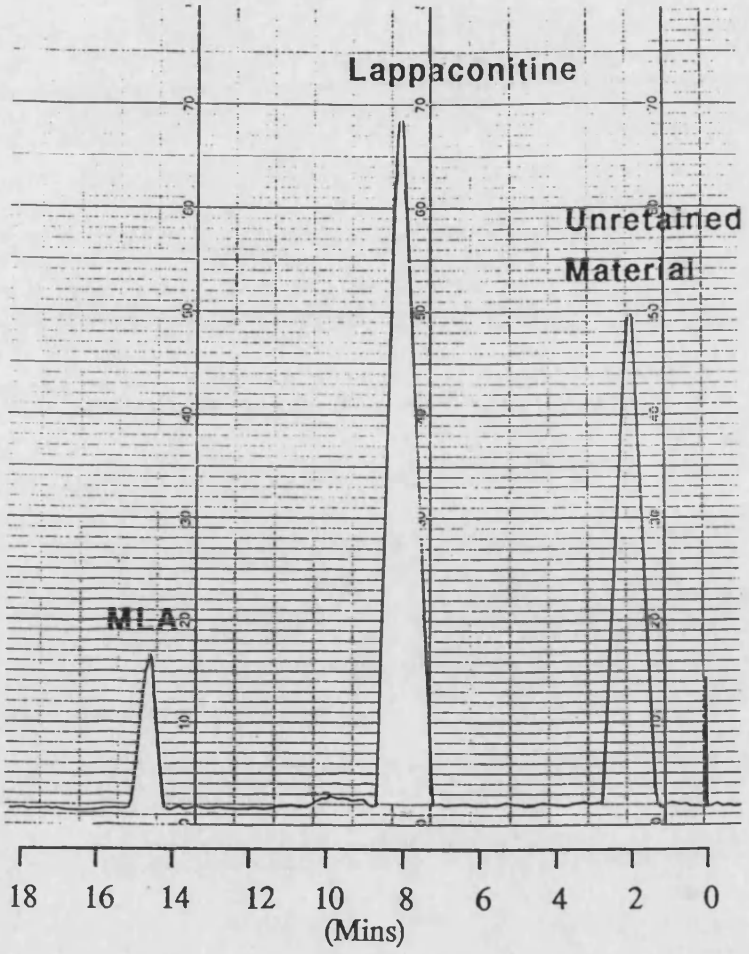
The optimization experiments (buffer pH and organic modifier content), led to the use of a mobile phase consisting of 0.2M ammonium acetate solution adjusted to pH 5 with formic acid-acetonitrile (70:30 v/v) [Figure (32)].

Calculation of α (selectivity) for MLA (1) and lappaconitine (39), using Equations I and IV in Section 3.2.4, with this optimum system, gave an average value of 2.01 which confirms good selectivity and indicates an appropriate choice of mobile phase. Calculation of R_S for the two alkaloids, using Equation V in Section 3.2.4.1, gave an average value of 5.3 which confirms, as can be seen by eye, that separation of the two components is complete.

Calculation of N, utilizing MLA (1) values for t_R and $w_{1/2}$ for this purpose (Equation III, Section 3.2.4.1), gave an average value of 2380 which indicates that the column is operating adequately (Hamilton and Sewell, 1986).

[See Section 3.3.2.3]

Figure (32) Separation of MLA (1) (10μg.cm^{-3}) and lappaconitine (39) (25μg.cm^{-3}) with 0.2M aqueous ammonium acetate solution (pH 5.0)-acetonitrile (70:30 v/v).



3.2.5 Calibration Curve

Calibrating a well resolved system with reliable standards leads to highly accurate analysis and thus means that the measured value to is very near to the true value (Hamilton and Sewell, 1986).

For the calibration of the determination of MLA (1) levels in other alkaloid samples, lappaconitine (39) was used as the internal standard. It was selected because it possesses an aromatic chromophore and in preliminary studies it was completely resolved from other alkaloid peaks, but eluted sufficiently near the peak of interest (MLA) using the chromatographic system decided upon. It was important that it did not react with any of the components, was not present in the original sample, was readily available in high purity, and was soluble in the mobile phase solvent. When constructing the calibration curve, it was added at a concentration which gave a similar peak height to MLA (1), having taken into account the different detector responses, if any.

The concentration of MLA (x values) and the peak heights were recorded. Peak heights were also measured for the known concentration of lappaconitine (39) present in the samples, and so peak height ratios [peak height of MLA (mm) / peak height of lappaconitine (mm)] were calculated (y values). These were computed by linear regression analysis (using "Minitab"). Thus, a "line of best fit" (plotting peak height ratio against MLA concentration) and the associated statistics were obtained.

Peak height measurements were used rather than peak area measurements because the former is more simple and accurate, especially for peaks displaying good symmetry (and for overlapping peaks) (Hamilton and Sewell, 1986).

The regression plot was found to be linear over the range of 2.0μg.cm^{-3} to 100μg.cm^{-3} and the regression equation was y = 16.717x + 0.0268 [Figure (33)]. The coefficient of determination (R^2) was 99.8% and the correlation coefficient (r) was 0.999, indicating a very high confidence level. The line had a slope of 16.7173 (standard deviation = 0.1361) and an intercept (constant) of 0.0268 (standard deviation = 0.005462). In this case, the standard deviation of the gradient indicates a high quality of data, but the standard deviation of the intercept is significant, being greater than three times the intercept of the ordinate axis, and therefore not passing exactly through the origin. This usually indicates that a small unidentified peak is inflating the size of the peak for the compound to be assayed. A sample of internal standard alone was therefore injected, in order to assess whether MLA (1) was concealing an interfering compound from the lappaconitine (39) sample, but this was found not to be the case.

A regression plot for the range of 0.2μg.cm^{-3} to 2.0μg.cm^{-3} had a correlation coefficient of only 0.915 and was unsuitable for accurate quantitation [Figure (34)], but a peak for MLA (1) was clearly and reproducibly detected down to approximately 50ng (70pmoles) on column (for a 0.5μg.cm^{-3} MLA solution). This lower limit of detection was taken to be the concentration that produced a signal having twice the amplitude of the random fluctuations about the baseline.

The lowest point on the linear section of the calibration curve [Figure (33)] (2.0μg.cm^{-3}) was equivalent to 200ng (290pmoles) of MLA on column. Manners and Pfister (1993) reported a normal-phase HPLC system with UV detection at 280nm, with a detection limit of 300ng on column. By using UV absorbance detection at 220nm they were able to detect 30ng on column, but this wavelength revealed that their chromatographic system did not fully resolve MLA (1) from its parent norditerpenoid alkaloid, lycoctonine (2). This effectively prevented them from using this shorter wavelength for the quantitative analysis of MLA.

[See Section 3.3.3]

Figure (33) Regression Plot over the range 2-100μg.cm^{-3} MLA (1)

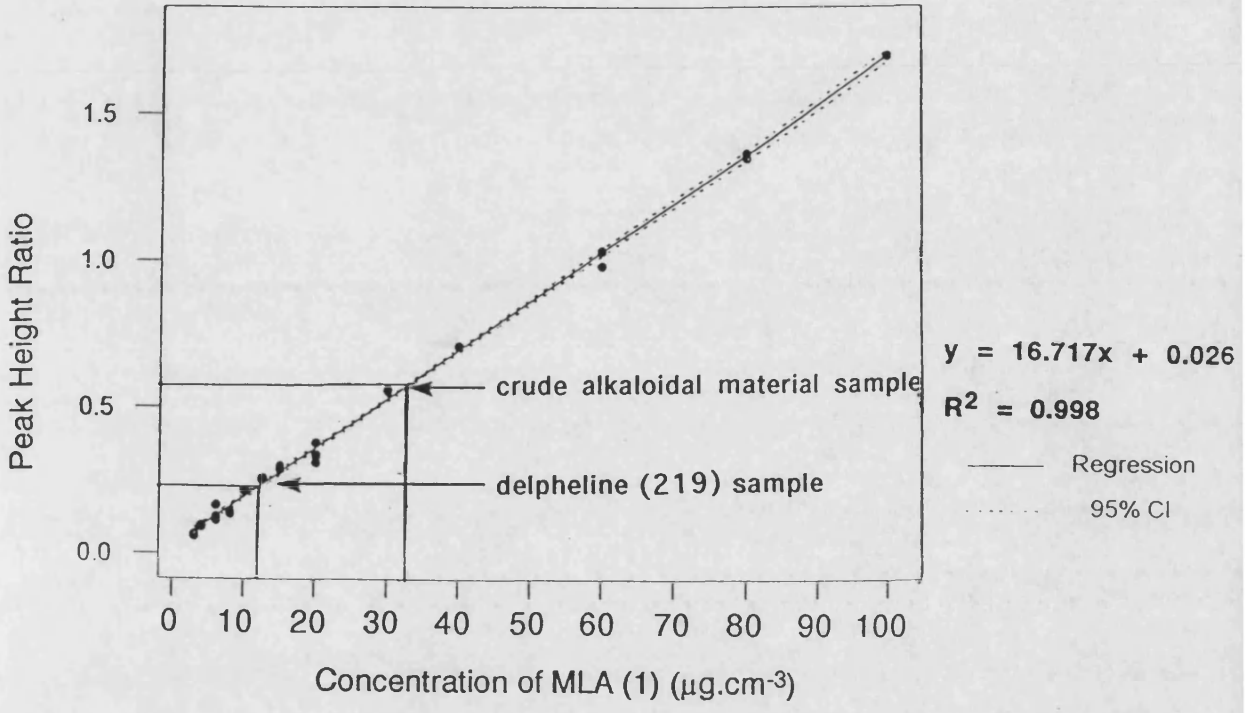
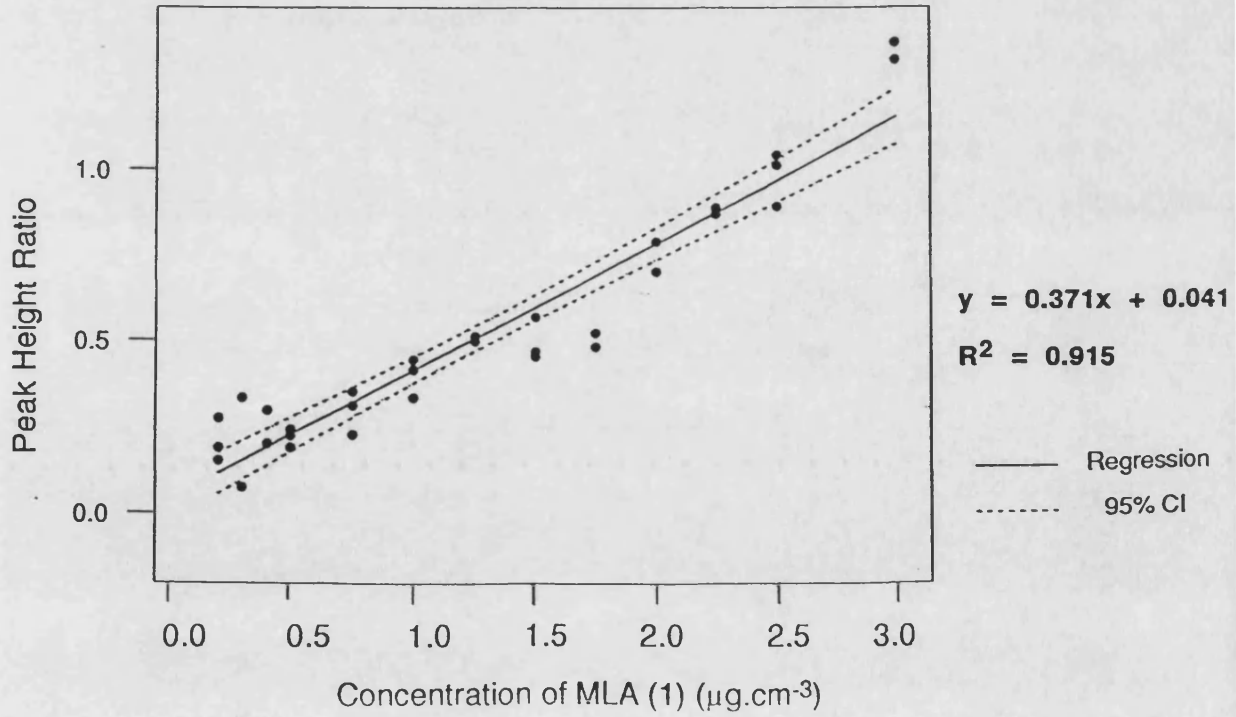


Figure (34) Regression Plot over the range 0.2-3.0μg.cm^{-3} MLA (1)



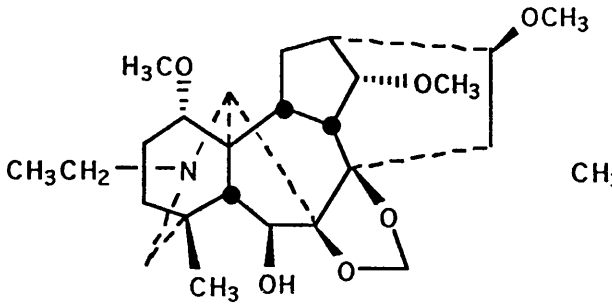
3.2.6 Relative Standard Deviation

In order to measure the precision of our optimized HPLC system, it was necessary to determine the systematic error on a series of six replicates at two different MLA (1) concentrations. For this purpose, the standard deviation (σ) was calculated and the relative standard deviation was then obtained from this, as a percentage by comparison with the mean ($100\sigma/x$), and found to be 5% at 5.0μg.cm^{-3} and 3.6% at 25μg.cm^{-3}. [See Section 3.3.4]

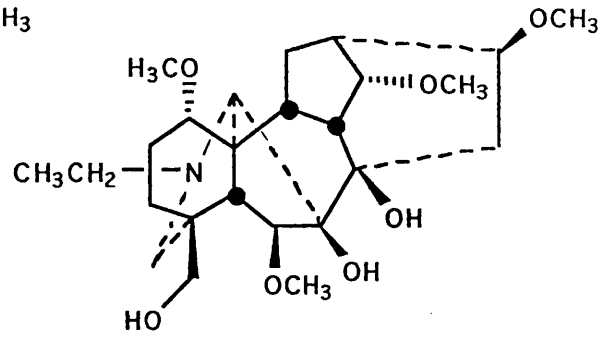
3.2.7 Analysis of Alkaloid Samples

The MLA (1) content in seeds of Garden Hybrid *Delphinium* was determined and the purity of isolated and semi-synthetic alkaloids was assessed using the calibration curve. The straight line calibration curve over the range 2.0μg.cm^{-3} to 100μg.cm^{-3} MLA [Figure (33)] was considered meaningful for our trace analysis, showing good accuracy of measurement. The samples to be measured for trace contamination, were injected on to the column in as large a volume as possible, but care was taken to avoid broad diffuse bands and overloading the column. The lengthy equilibration time (45mins) required to return the column to its original condition, in our studies, demonstrated one of the drawbacks of trace analysis.

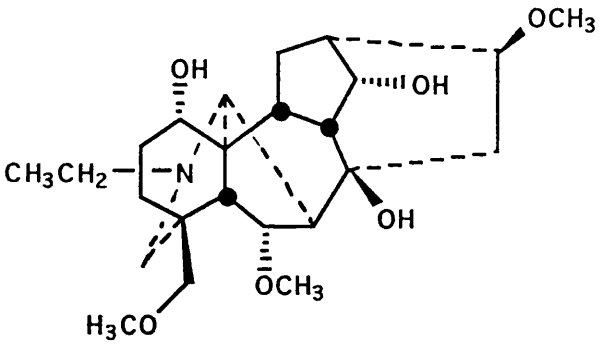
For the analysis of the alkaloid samples, mixtures consisting of 5-9mg.cm^{-3} alkaloid and 25μg.cm^{-3} of lappaconitine (39) in mobile phase were injected. Peak height ratios were calculated and MLA (1) concentration read from the calibration curve. Using UV detection at 270nm, only those alkaloids possessing an aromatic acyl group were detected therefore on injection of the delpheline (219), lycoctonine (2), and neoline (246) samples, no additional peaks were observed.



(219) Delpheline



(2) Lycoctonine



(246) Neoline

For the crude alkaloidal material, an average peak height ratio of 0.59 correlates to a MLA (1) concentration of 33.7μg.cm^{-3} [Figure (35)]. Thus, the 60μg.cm^{-3} sample of crude total alkaloid contains 563μg MLA per mg [56.3% ($^{W}/_{W}$) MLA]. (The general method for calculating the level of MLA in the alkaloid samples is shown below, using the figures for delpheline). MLA therefore represents 0.70% of the dry weight of Garden Hybrid *Delphinium* seeds, based on a yield of 1.25% for the total alkaloid mixture obtained on extraction of seeds [Section 2.3.2.1].

Figure (35) also shows that the sample of crude alkaloidal material contains another alkaloid with an aromatic chromophore similar to that in MLA (detectable by UV absorbance at 270nm), with t_R = 15.5mins. From this chromatogram, it is not possible to either identify this alkaloid or to estimate the percentage of it in the mixture (because it is likely that this alkaloid exhibits a different UV absorption maximum).

For the lycoctonine (2) and neoline (246) samples, no MLA peaks were seen. Using the observation that 0.5μg.cm^{-3} MLA (1) is the lowest concentration reproducibly detected, we can deduce that the 2600μg.cm^{-3} lycoctonine (2) sample contained less than 0.192μg MLA per mg of lycoctonine (2) which equates to 0.019% ($^{W}/_{W}$) or 0.014moles% MLA. For the delpheline (219) sample, an average peak height ratio of 0.23 correlates to a MLA (1) concentration of 12.3μg.cm^{-3} [Figure (36)]. We deduce that the 4400μg.cm^{-3} sample contains 2.79μg MLA per mg of delpheline (219). This is equivalent to 0.28% ($^{W}/_{W}$) and 0.18moles% MLA. Similarly, it can be said that the MLA (1) content of the 3000μg.cm^{-3} neoline (246) sample is less than 0.017% ($^{W}/_{W}$) [0.011moles%].

Figure (35) Crude Alkaloidal Material (60μg.cm^{-3}) and lappaconitine (39) (25μg.cm^{-3}) with 0.2M aqueous ammonium acetate solution (pH 5.0)-acetonitrile (70:30 v/v).

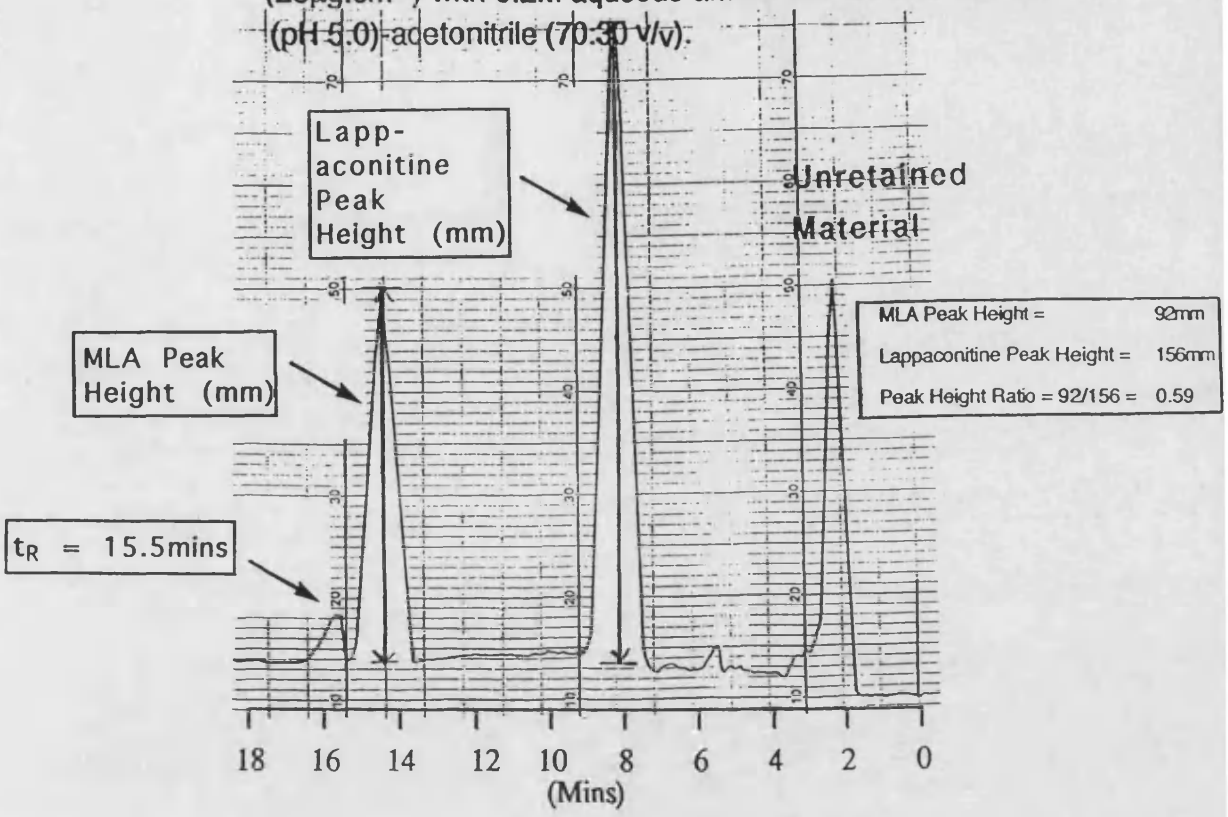
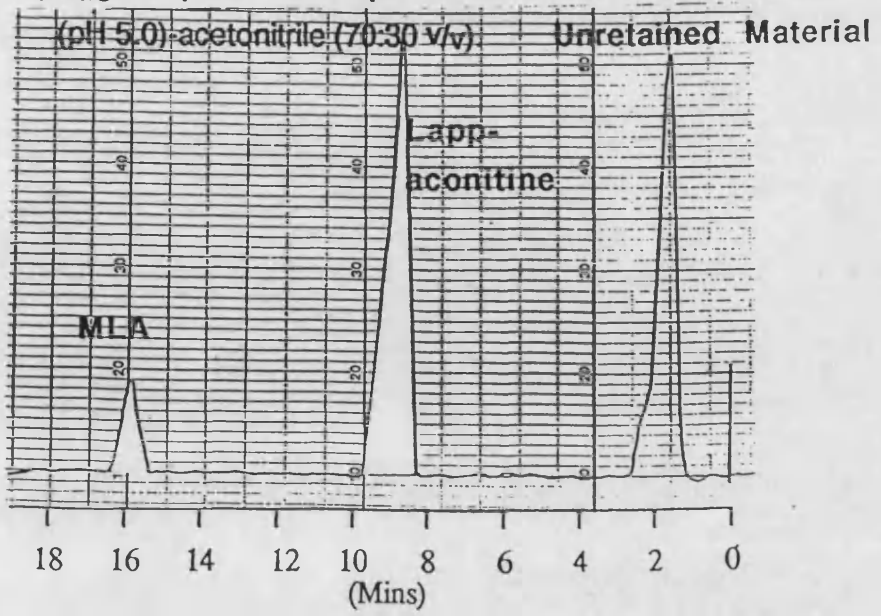


Figure (36) Delpheline (219) (4400μg.cm^{-3}) and lappaconitine (39) (25μg.cm^{-3}) with 0.2M aqueous ammonium acetate solution (pH 5.0)-acetonitrile (70:30 v/v).



Mass of alkaloid taken to make up the original sample:	8.8mg
Concentration of alkaloid after dissolving in 1cm^3 mobile phase and mixing with 1cm^3 lappaconitine solution:	4400$\mu g.cm^{-3}$
Mass of alkaloid injected:	440μg
MLA peak height measured:	26mm
Lappaconitine peak height measured:	111mm
Peak height ratio = peak height of MLA (mm)/peak height of lappaconitine (mm) =26/111 =	0.23
Concentration of MLA in the injection sample (from the calibration curve, peak height ratio of 0.23 correlates with):	12.3$\mu g.cm^{-3}$
Mass of MLA detected in injection sample (100μl loop):	1.23μg
% MLA in the injection sample ($^{w}/_{w}$) = 100 x mass of MLA in the injection sample x [total sample volume/ injected sample volume]/mass of alkaloid in the original sample = 100 x 1.23μg x [2cm^3/100 x $10^{-3}cm^3$]/8800μg =	2.8%
moles% MLA in the injection sample = 100 x [mass of MLA in the injection sample/molecular weight of MLA]/ [mass of alkaloid in the injection sample/molecular weight of alkaloid] = 100 x [1.23μg/682]/[440μg/449] =	0.18moles%

[See Section 3.3.5]

3.2.8 LC/MS

3.2.8.1 Rationale

Many of the norditerpenoid alkaloids in our investigations do not have a specific (strong) chromophore therefore we decided to use thermospray mass spectroscopy as an alternative HPLC detection method to UV absorption. The aims were to develop a system which is generally applicable to alkaloids of this type irrespective of their UV absorbing properties and to use the technique both

for the screening of *Delphinium* extracts for these alkaloids and for monitoring the purity of alkaloid samples prior to biological testing.

To date, there has been only one report of LC/MS determination of C_{19}- and C_{20}-diterpenoid alkaloids with poor UV absorption [Wada *et al.*, 1993 (See Section 3.2.1)]. However, an extensive review on the analysis of alkaloids in general by LC/MS published by Verpoorte and Niessen (1994) illustrates the success of the on-line combination of LC and MS. The coupling of thermospray mass spectroscopy with LC is of particular interest because not only is it a technique with a sensitive (high picogram range) and selective detection method, but the compatibility of the thermospray nebulizer with conventional HPLC conditions results in the suitability of this widely used interface with the relatively high flow rate ($1cm^3.min^{-1}$) of a mobile phase with a high water content (70%), as used in our reverse-phase method (Verpoorte and Niessen, 1994). In addition, LC/MS offers the possibility of identifying components of overlapping peaks, provided that they have different molecular weights.

3.2.8.2 Theory of Thermospray MS

The working principle of the thermospray technique involves a proportion of the LC effluent being taken into the ion-source *via* a narrow bore tube inlet, which is surrounded by a heated copper block (Gilbert, 1987). Vaporization of the eluant just as it reaches the exit of the capillary tube is achieved by rapid heating of the block, producing a superheated mist in a supersonic jet of vapour into a region of reduced pressure (nebulization). The vaporizer temperature is primarily determined by the mobile phase composition and flow rate and must be adjusted to obtain almost complete evaporation of the column effluent inside the vaporizer capillary (Verpoorte and Niessen, 1994). The volatility of an analyte and the temperature above which thermal decomposition occurs are therefore factors which determine suitability for thermospray. An interesting aspect of the technique is that the volatilized solvent then becomes a chemical

ionization (CI) reagent gas (solvent-mediated, gas-phase CI) thus often no external ionizing device is necessary. This is especially the case with eluates containing dilute, volatile buffers (for example, as used in our investigations, ammonium acetate and formic acid). In our studies, positive ion thermospray ionization was used. Thus, proton transfer from positively charged solvent molecules, either by a spontaneous ionization process (ion evaporation) or induced by electrons from a heated filament or a discharge electrode, results in ionization of the sample molecules. After these ion-molecule reactions and when the solvent evaporates from the droplets (droplet desolvation), cationized molecules e.g. $[M+H]^+$ and $[M+NH_4]^+$ ions may remain in the gas phase to be recorded. Electron induced ionization is more prone to producing fragmentation, but usually there is very little fragmentation as a result of these soft ionizations (Verpoorte and Niessen, 1994). Ionization and fragmentation is influenced to a large degree by the mobile phase composition and for most applications for thermospray buffer ionization, the percentage of organic modifier must be below 40% otherwise a decrease in sensitivity is observed. Also, the buffer concentration must be higher than the analyte concentration (Verpoorte and Niessen, 1994). The most frequently used electrolytes for the thermospray ionization are ammonium acetate and ammonium formate, but other common volatile buffer systems include ammonium carbonate, ammonium bicarbonate, and triethylamine.

The solvent molecules are removed from the ion-source by fast pumping, whereas the sample ions are extracted from the jet by an electric field. In our studies, a repeller electrode, positioned opposite the ion sampling aperture of the mass analyzer, with a potential of 100volts was used. The alternative is a retarder electrode positioned slightly downstream. The potential of the electrode influences not only the total ion current and the abundance of the various ionic species from the analyte molecules (higher potentials induce fragmentation of analyte), but also the various adducts with m/z 15-100 generated from the mobile phase (Verpoorte and Niessen, 1994).

Most LC/MS work is performed using a high-resolution magnetic sector instrument or a quadropole MS equipped with a vacuum system suitable for CI operation, but tandem mass spectrometers are also adaptable (Gilbert, 1987).

3.2.8.3 LC/MS of Alkaloid Samples

LC/MS was used to identify and confirm the identity of the major alkaloids in the seeds of Garden Hybrid *Delphinium* and to verify the identity and establish the purity of isolated and semi-synthetic alkaloids. For each of the samples, the major HPLC peaks obtained using the optimized LC system, were used to provide the m/z values of the molecular ion peaks (protonated molecule ($[M+H]^+$) in the mass spectra, by monitoring the total ion current between m/z 400 and m/z 751. [See Section 3.3.6]

Table 6 shows the retention time and corresponding molecular ion and ion fragments, where applicable, obtained for each of the alkaloid samples. These diagnostic peaks were used to give structural information.

Table 6

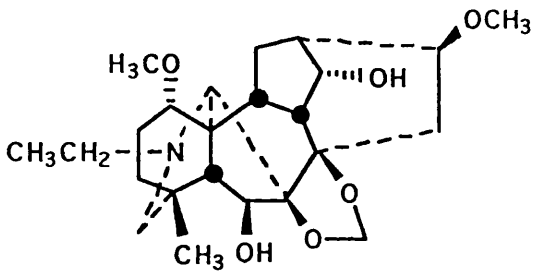
LC/MS analysis of Alkaloid Samples

Alkaloid	Concn of alkaloid injected ($\mu g.cm^{-3}$)	Peak t_R (mins)	Ion(s) (m/z)
Crude Alkaloidal Material	100	2.5	394
			422
			454
		3.5	436
		5.5	450
		7.0	669
		10.5	653
			698
		12.5	683
		14.5	711

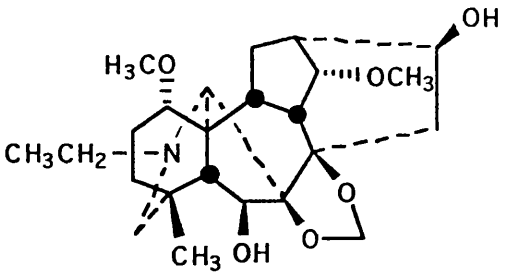
Table 6 cont.

Alkaloid	Concn of alkaloid injected ($\mu g.cm^{-3}$)	Peak t_R (mins)	Ion(s) (m/z)
MLA (1)	100	12.5	683
		14.5	711
Lycoctonine(2)	100	2.5	454
		3.25	468
		5.5	450
Delpheline (219)	100	3.5	436
		5.0	450
Neoline (246)	100	3.0	438
			454

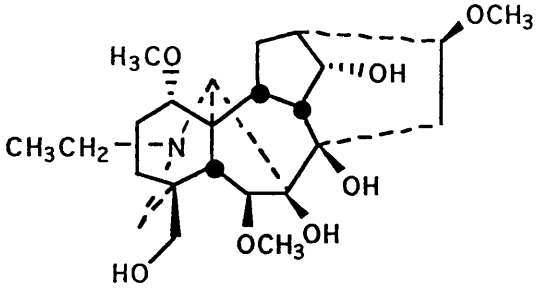
Figure (37) shows details of the data in **Table 6** for the crude alkaloidal material, in the form of the total ion chromatogram for the crude alkaloid mixture, together with selected ion chromatograms corresponding to $[M+H]^+$ ions for each of the major peaks. These reveal important components with m/z 436, 450, 669, 683, and 711. In addition, the small first-eluting peak (with t_R = 2.5mins) proved to be a complex mixture with prominent ions at m/z 394, 422, and 454 which may be highly polar alkaloids plus other peaks which are probably non-alkaloidal and the very minor peak with t_R = 10.5mins indicated the presence of alkaloids with molecular ions at m/z 653 and 698, the former of which is consistent with the structure of anhweidelphine (33). The peak with m/z 436 (t_R = 3.5mins) can be tentatively identified as delelatine (252) or eladine (253), both of which have been reported from *Delphinium elatum* L., a species which figures strongly in the ancestry of Garden Hybrid *Delphinium* (Cookson and Trevett, 1956). *D. elatum* (Pelletier and Joshi, 1991 and Pelletier *et al.*, 1984) is also known to contain, amongst other alkaloids, delpheline (219), isodelpheline (220), MLA (1), nudicauline (37), 14-deacetylnudicauline (38), elatine (32), lycoctonine (2), elasine, and deltaline.



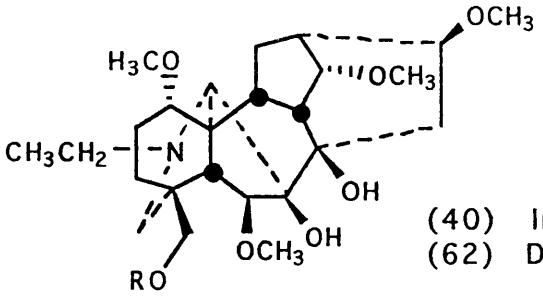
(252) Delelatine



(253) Eladine

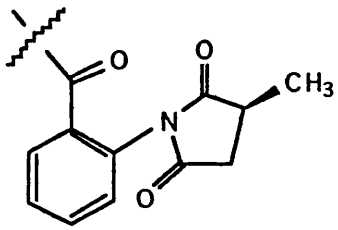


(254) Delectinine

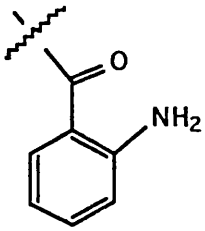


(40) Inuline
(62) Delsemine

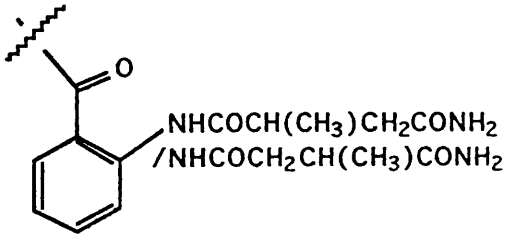
For (31), (32), (33), (37) and (38)
R =

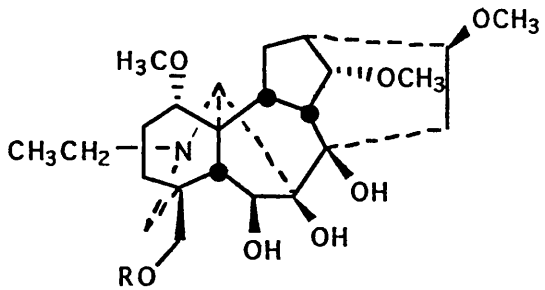


For (40)
R =

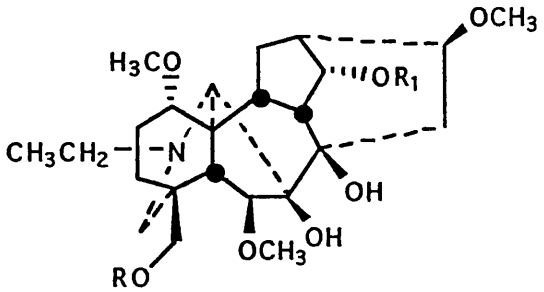


For (62)
R =

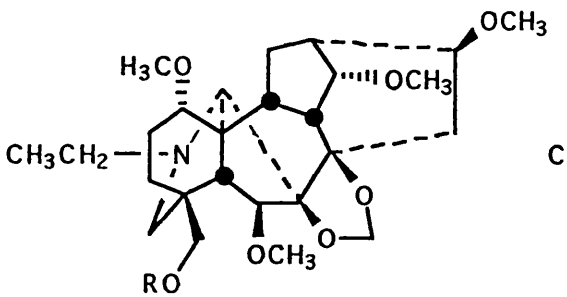




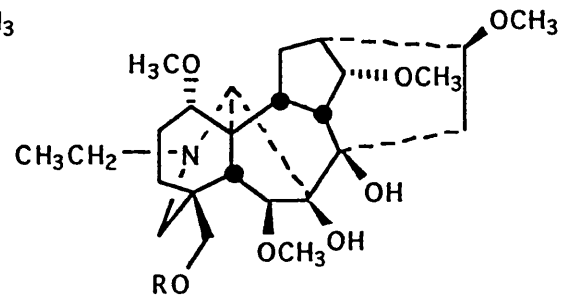
(31) Glaudelsine



(37) Nudicauline R_1 = Ac
(38) 14-Deacetylnudicauline R_1 = H



(32) Elatine

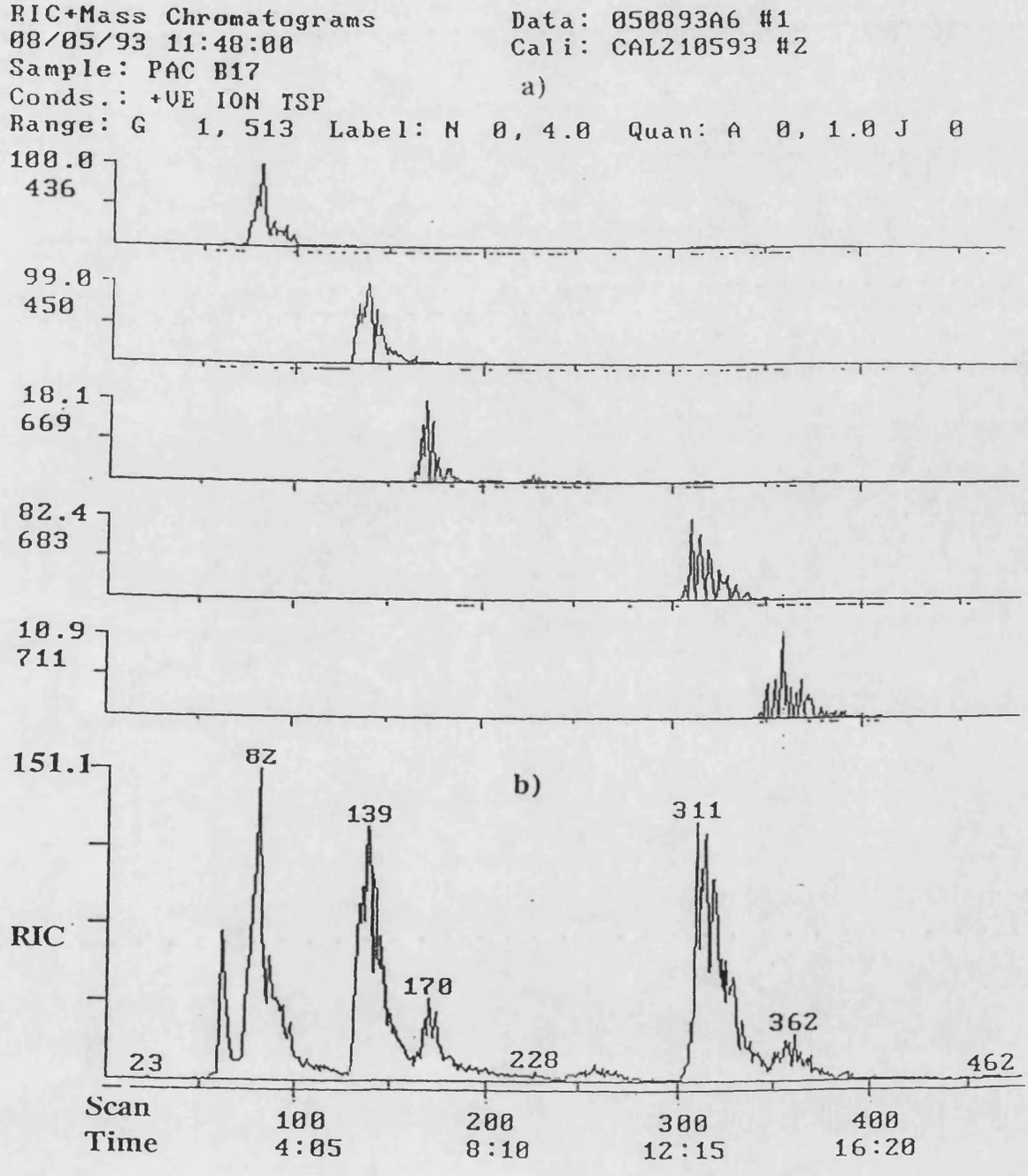


(33) Anhweidelphinine

Figure (37) LC/MS Analysis of Crude Alkaloidal Material Sample.

a) Selected Ion Chromatograms showing components with m/z 436, 450, 669, 683, and 711.

b) Total Ion Chromatogram.



Thus, the peak with $^{m}/_{z}$ 450 and t_R = 5.5mins can be established as either delpheline (219) or isodelpheline (220) and the identity of the MLA (1) ($^{m}/_{z}$ 683) (t_R = 12.5mins) peak can be confirmed. MLA (1) can be seen to be the most abundant compound detected, followed by the alkaloids with molecular weights 449 and 435. The minor peak with $^{m}/_{z}$ 669 (t_R = 7.0mins) is consistent with the structure of 14-deacetylnudicauline (38) or glaudelsine (31), the latter of which is an alkaloid from *Delphinium glaucescens* Rydb. which has recently attracted much attention (Kukel and Jennings, 1994). Another structurally diagnostic ion at $^{m}/_{z}$ 711 was indicative of the presence lesser amounts of nudicauline (37) (t_R = 14.5mins) [a known contaminant in many samples of MLA (Majak *et al.*, 1987)]. There was also evidence for almost insignificant amounts of elatine (32) ($^{m}/_{z}$ 694), elasine ($^{m}/_{z}$ 494), deltaline ($^{m}/_{z}$ 507), delsemine (62) ($^{m}/_{z}$ 700), and inuline (40) ($^{m}/_{z}$ 587) in the crude alkaloid mixture. Paciline ($^{m}/_{z}$ 463) and pacinine ($^{m}/_{z}$ 447), which are known in *Delphinium pacific giant* Mix, a closely related hybrid to the garden hybrid used in our studies, were interestingly not detected in the crude mixture by this procedure.

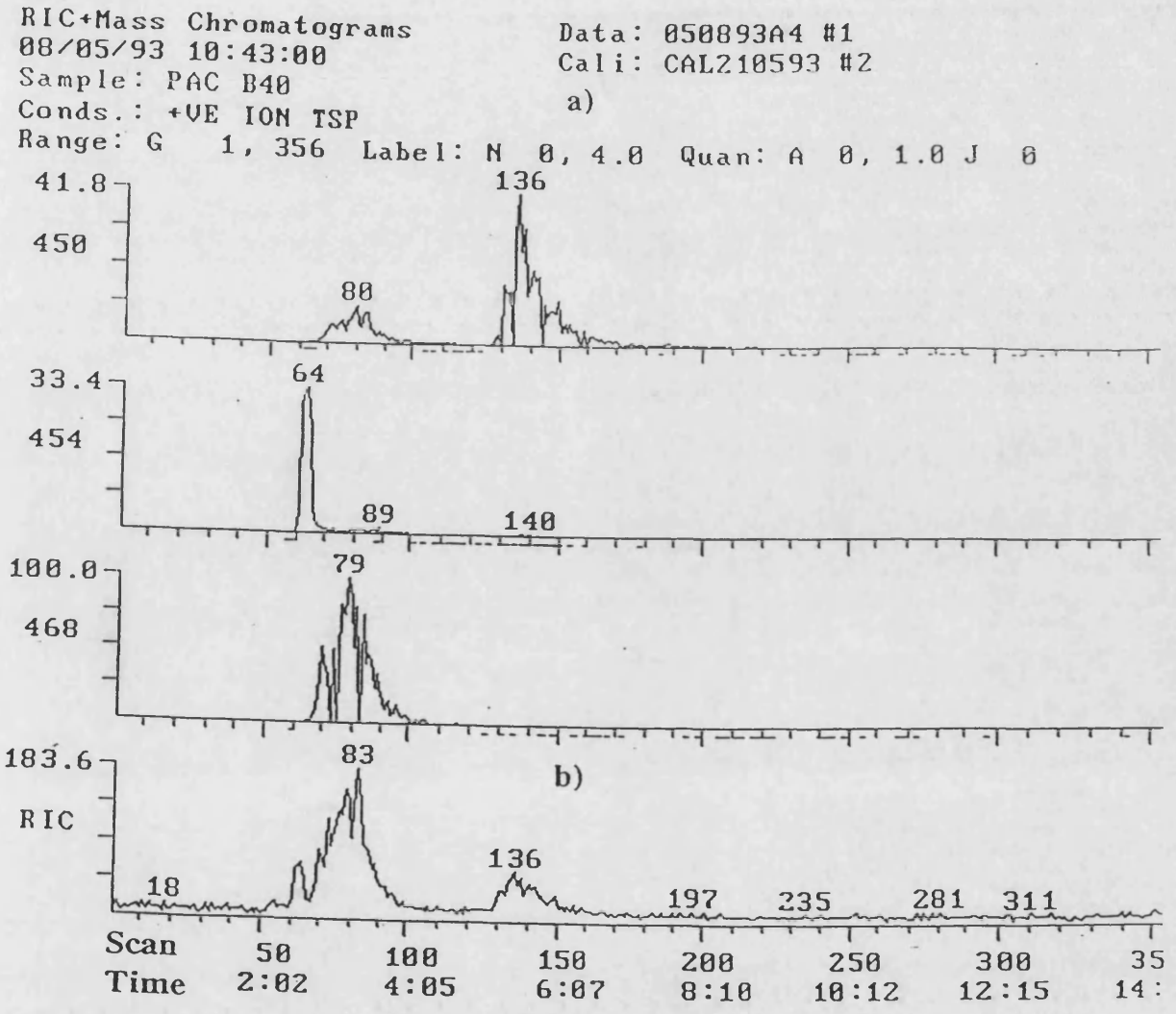
The MLA (1) sample revealed the major component to have $^{m}/_{z}$ 683 (t_R = 12.5mins), as expected (See Section 2.3.2.6). In addition, the presence of a minor component is indicated by $^{m}/_{z}$ 711 (t_R = 14.5mins), as seen in the crude seed extract, which is consistent with the molecular weight of nudicauline (37). Nudicauline (37) is known in *Delphinium nudicaule* Torr and Gray and *Delphinium nuttalianum* Pritz, as well as *Delphinium elatum* L. and MLA (1) is found in many *Delphinium* species including *Delphinium elatum* L (Pelletier and Joshi, 1991 and Pelletier *et al.*, 1984).

Figure (38) shows details of the data in **Table 6** for the lycoctonine (2) sample (See Section 2.3.2.7), in the form of the total ion chromatogram, together with selected ion chromatograms corresponding to $[M+H]^+$ ions for each of the major peaks. These reveal that the lycoctonine (2) sample predominantly consists of

Figure (38) LC/MS Analysis of lycoctonine (2) Sample.

a) Selected Ion Chromatograms showing components with m/z 450, 454, and 468.

b) Total Ion Chromatogram.



the component with m/z 468 (t_R = 3.25mins) as anticipated but also contains important components with 454 (t_R = 2.5mins) and m/z 450 (t_R = 5.5mins). Thus, it is proposed that the lycoctonine (2) sample contains a trace of one of the more polar alkaloids observed the total crude extract, as well as significant amounts of delpheline (219) or isodelpheline (220). From the absence of an ion at m/z 468 corresponding to this peak in the crude alkaloid mixture, we conclude that the seeds of Garden Hybrid *Delphinium* contain no lycoctonine (2), despite its occurrence in many *Delphinium* species (as well as *Aconitum* and *Consolida*), including *Delphinium elatum* L. It should be noted that, chromatographically, lycoctonine (2) is not resolved from delelatine (252) or eladine (253) (t_R = 32.5-34.5mins), but can be readily distinguished by its spectral data.

Unlike the peak with retention time 2.5mins in the crude alkaloid mixture, the similar peak in the lycoctonine (2) sample showed a single mass spectral peak at m/z 454, consistent with delectinine (254) [14-*O*-demethyl lycoctonine or the parent alcohol of 14-deacetylnudicauline (38)] (t_R = 2.5mins), known in *Delphinium andersonii* Gray and *Delphinium dictyocarpum* DC (**Table 6**).

Figure (39) shows details of the data in **Table 6** for the delpheline (219) sample (See Section 2.3.2.2), in the form of the total ion chromatogram, together with the mass spectrum of a minor component (t_R = 3.5mins) revealing m/z 436 and the mass spectrum of the major component (t_R = 5.0mins) revealing m/z 450. It is not possible to determine whether the alkaloid with molecular weight 449 is delpheline (219) (which is known in *Delphinium pacific giant* Mix, *Delphinium elatum* L., *Delphinium barbeyi* Huth, *Delphinium occidentale* S. Wats., and *Delphinium ternatum* Huth) or isodelpheline (220) (from *Delphinium elatum* L.). It is clear that the delpheline (219) sample contains only trace quantities of the alkaloids proposed as delelatine (252) or eladine (253). Delelatine (252) has been isolated from *Delphinium elatum* L. and

Delphinium tatsienense Franch., while eladine (253) has been found in *Delphinium elatum* L. only.

Figure (39) a) and b) illustrate the appearance of peaks at one mass unit higher for singly charged ions in the mass spectrum. This results from the natural abundance of ^{13}C (1.1%), thus for an ion containing 25 carbon atoms, the abundance of the isotope peak is 27.5% of the ^{12}C-containing peak. Obviously the probability of finding two ^{13}C atoms in an ion is very low for smaller molecules but for larger molecules, such as diterpenoid alkaloids, a peak at two mass units higher than the ion containing ^{12}C only is occasionally observed, but only at low abundance.

Figure (40) shows details of the data in **Table 6** for the neoline (246) sample, in the form of the total ion chromatogram, together with the mass spectrum of the component with retention time of 3.0mins, revealing m/z 438 and m/z 454 (the latter being a minor peak). Hence, the neoline (246) sample is believed to contain trace amounts of a compound with an additional oxygen atom, possibly 15α-hydroxyneoline and/or 15β-hydroxyneoline (m/z 454), which have been reported in many *Aconitum* species as is neoline itself. Chromatographically, neoline (246) appears not to be resolved from the 15-hydroxy substituted compounds, but can be readily distinguished by its spectral data. 15α-Hydroxyneoline can also known as fuziline and senbusine C and 15β-hydroxyneoline can also be called crassicaulisine or nagarine.

For all the samples, the conditions used gave the $[M+H]^+$ ion as the predominant ion with little fragmentation, therefore MS/MS could be a very powerful technique to reveal further structural information. This technique is performed with two mass spectrometers in tandem, such that the molecular ion from an initial mass spectrum is selected and made to give a second mass spectrum. Nonetheless it has been demonstrated that the LC/MS method is a valuable technique for the separation and simultaneous determination of

Figure (39) LC/MS Analysis of delpheline (219) Sample.

a) Mass Spectrum of Minor Component (t_R = 3.5mins) revealing m/z 436.

b) Mass Spectrum of Major Component (t_R = 5.0mins) revealing m/z 450.

c) Total Ion Chromatogram.

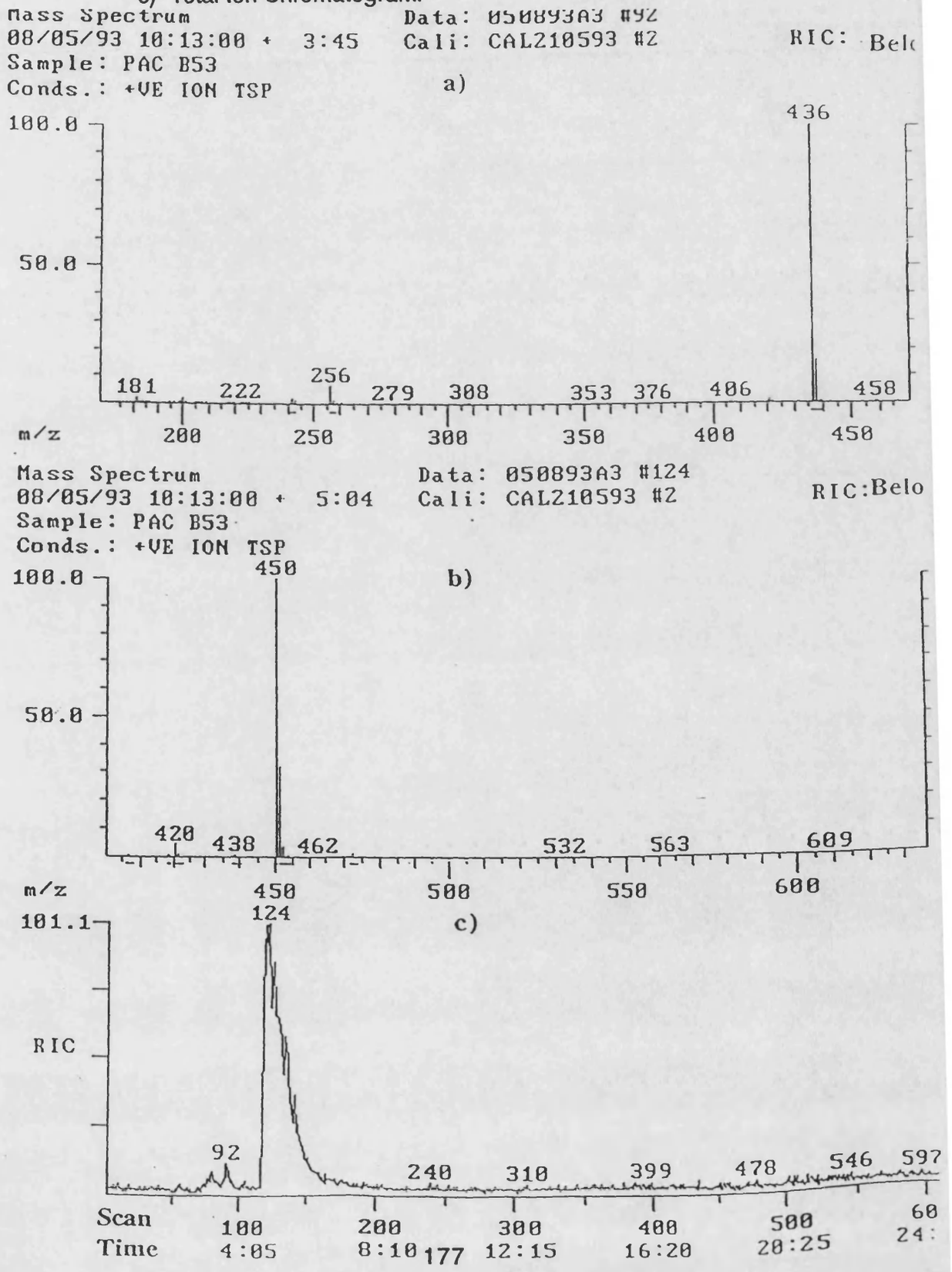
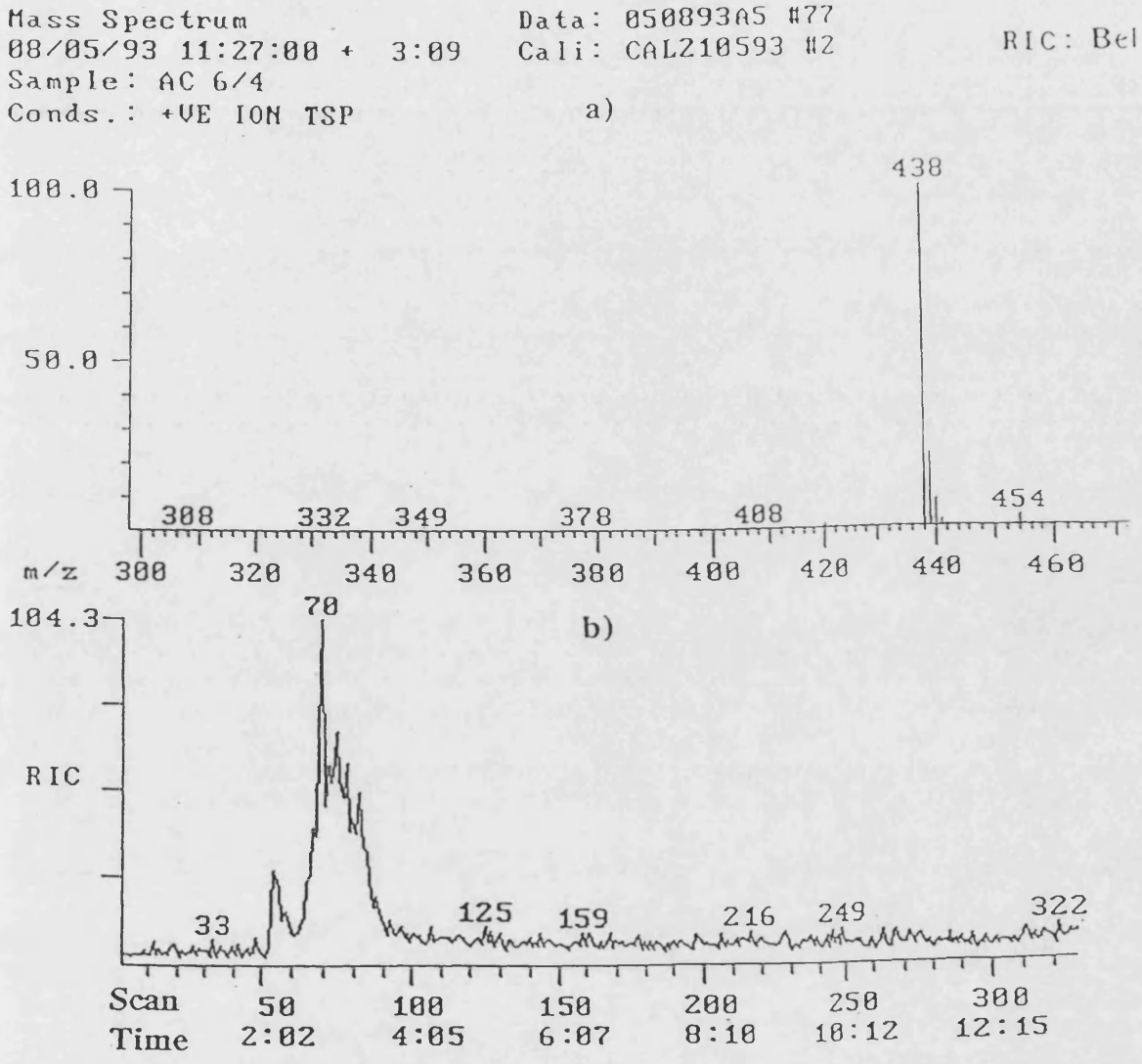


Figure (40) LC/MS Analysis of neoline (246) Sample.

a) Mass Spectrum revealing m/z 438 and m/z 454 (t_R = 3.0mins).

b) Total Ion Chromatogram.



norditerpenoid alkaloids in *Delphinium* seed extracts and for monitoring the purity of isolated alkaloid samples prior to biological testing or chemical manipulation. This procedure is applicable to the determination of the alkaloid content of small samples of plant material.

3.2.9 Chromatographic Considerations

TLC analysis of a sample is often used as a first indication of the correct HPLC operating conditions (silica gel plates for normal-phase columns and silylated silica gel plates for reverse-phase columns) (Hamilton and Sewell, 1986), but the order of elution is not always consistent with a prediction based on TLC, especially for liquid-liquid chromatography (LLC) (whereby the solute molecules are partitioned between the mobile liquid phase and the stationary liquid phase). It is believed that some discrepancies of this type arise when using this prediction method as a result of the fact that the surface areas of silica gels employed for TLC are about twice those used in column packings and for this purpose the R_f should be <0.3 (Marston and Hostettmann, 1991). It is often found that TLC is more sensitive to differences in polarity and less so to molecular weight differences between solute species, whereas the converse is true for LLC, but sometimes, as shown in this case (**Table 7a** and **7b**), these trends are not clear (Hamilton and Sewell, 1986).

Table 7a shows that for the alkaloids, delectinine, neoline, lycoctonine, delelatine / eladine and delpheline, the number of polar hydroxyl groups seems to have an important effect on their retention,with the rough trend being for R_f to decrease and κ' to increase as the number of hydroxyl groups decreases (that is, for less polar solutes to be eluted first in a normal-phase system and last in a reverse-phase system). However, the high R_f value obtained for delpheline (219) does not fit this trend and cannot be explained by its molecular weight or the number of carbon atoms either. Infact MW and number of C atoms seem to

have little bearing on R_f within this set, but it is possible to establish a trend for their effect on κ'.

For 14-deacetylnudicauline / glaudelsine, MLA and nudicauline (**Table 7b**) disregarding lappaconitine), the trends seems more orientated towards the effect of the molecular weights or number of carbons atoms on R_f and κ', such that R_f decreases and κ' increases with increasing molecular weight or the number of C atoms. However, it could equally be argued that the R_f values are being controlled by the number of -OH groups, while any trend related to κ' is difficult to interpret.

As soon as members of the two groups are compared to members outside its group, then the story gets more complicated. For example, it might be expected that lycoctonine (2) would give an R_f value greater than that for MLA (2), owing to its additional polar OH group, but infact by TLC lycoctonine runs slower than MLA. It should be noted that the κ' value for lycoctonine is smaller than that of MLA, but it is difficult to predict whether this is dictated by polar groups or MW.

Table 7a

Summary of Relationships between Retention Data and Structure of Alkaloids

Alkaloid	R_f *	κ'	Number of -OH groups	MW	Number of C atoms
Delectinine (254)	-	~0.5	4	453	24
Neoline (246)	0.2	0.8	3	437	24
Lycoctonine (2)	0.2	1.0	3	467	25
Delelatine (252) /Eladine (253)	0.15	~0.9	2	435	24
Delpheline (219)	0.45	2.3	1	449	25

Table 7b

Alkaloid	R_f *	κ'	Number of -OH groups	MW	Number of C atoms
Deacetylnudicauline (38) /Glaudelsine (31)	-	~2.9	3	668	36
MLA (1)	0.3	6.9	2	682	37
Nudicauline (37)	0.25	7.3	2	710	38
Lappaconitine (39)	0.4	3.4	2	584	32

* TLC data was obtained using cyclohexane-chloroform-diethylamine 5:4:1 on silica gel.

3.3 EXPERIMENTAL

3.3.1 Instrumentation and Experimental Techniques

3.3.1.1 Solvents and Reagents

Lappaconitine (39) was purchased from Latoxan (Rosans, France) or BioSpecs (The Hague, The Netherlands). Neoline (246) was a gift from Chen Si Ying (Kunming Institute of Botany, Kunming 650204, Yunnan, P. R. China), isolated from *Aconitum carmichaeli* Debaux. Reagent grade ammonium acetate and formic acid, and HPLC grade acetonitrile were used in the preparation of the mobile phase. Water refers to double distilled deionized water.

a) Mobile Phase

The buffer solutions were prepared by dissolving ammonium acetate (15.416g, 0.2mol) in water (~950cm^3), acidifying to the desired pH (3.0 to 5.0 with formic acid) and then making the mixture up to 1000cm^3 with water. This aqueous component of the mobile phase was filtered under reduced pressure through cellulose filter paper and then combined with acetonitrile to give the desired percentage composition. The mobile phase was degassed using a helium sparge or an ultrasonic bath (15mins).

b) Alkaloid Samples

i) Stock Solutions

MLA (1) (4.5mg) was dissolved in mobile phase (4.5cm^3). 1 in 5 dilution of this 1.0$mg.cm^{-3}$ solution gave Solution A (200$\mu g.cm^{-3}$ MLA). 1 in 10 dilution of Solution A gave Solution B (20$\mu g.cm^{-3}$ MLA). 1 in 10 dilution of Solution B gave Solution C (2.0$\mu g.cm^{-3}$ MLA). 1 in 20 dilution of the original 1.0$mg.cm^{-3}$ MLA

solution gave Solution D (50μg.cm^{-3} MLA). 1 in 2 dilution of Solution D gave Solution E (25μg.cm^{-3} MLA). 1 in 5 dilution of Solution E gave Solution F (5.0μg.cm^{-3} MLA).

Lappaconitine (39) (2.5mg) was dissolved in mobile phase (5cm^3). 1 in 10 dilution of this 500μg.cm^{-3} solution gave Solution G (50μg.cm^{-3} lappaconitine). 1 in 20 dilution of Solution G gave Solution H (2.5μg.cm^{-3} lappaconitine).

ii) Calibration Mixtures

The series of solutions containing MLA (1) and lappaconitine (39) for the construction of a calibration curve, was prepared as shown in **Table 8.**

Table 8

Preparation of Calibration Mixtures

Volume MLA (Solution) (cm^3)	Volume Lappaconitine (Solution) (cm^3)	Volume Mobile Phase (cm^3)	Resulting Concentrations (μg.cm^{-3})	
			MLA	Lappaconitine
0.5 (A)	0.5 (G)	-	100	25
0.4 (A)	0.5 (G)	0.1	80	25
0.3 (A)	0.5 (G)	0.2	60	25
0.2 (A)	0.5 (G)	0.3	40	25
0.15 (A)	0.5 (G)	0.35	30	25
0.1 (A)	0.5 (G)	0.4	20	25

Table 8 cont.

Volume MLA (Solution) (cm^3)	Volume Lappaconitine (Solution) (cm^3)	Volume Mobile Phase (cm^3)	Resulting Concentrations ($\mu g.cm^{-3}$)	
			MLA	Lappaconitine
0.3 (D)	0.5 (G)	0.2	15	25
0.25 (D)	0.5 (G)	0.25	12.5	25
0.5 (B)	0.5 (G)	-	10	25
0.4 (B)	0.5 (G)	0.1	8.0	25
0.3 (B)	0.5 (G)	0.2	6.0	25
0.2 (B)	0.5 (G)	0.3	4.0	25
0.15 (B)	0.5 (G)	0.35	3.0	25
0.1 (B)	0.5 (G)	0.4	2.0	25

Table 8 cont.

Volume MLA (Solution) (cm^3)	Volume Lappaconitine (Solution) (cm^3)	Volume Mobile Phase (cm^3)	Resulting Concentrations ($\mu g.cm^{-3}$)	
			MLA	Lappaconitine
0.15 (B)	0.5 (H)	0.35	3.0	1.25
0.5 (F)	0.5 (H)	-	2.5	1.25
0.45 (F)	0.5 (H)	0.05	2.25	1.25
0.4 (F)	0.5 (H)	0.1	2.0	1.25
0.35 (F)	0.5 (H)	0.15	1.75	1.25
0.30 (F)	0.5 (H)	0.20	1.5	1.25
0.25 (F)	0.5 (H)	0.25	1.25	1.25
0.50 (C)	0.5 (H)	-	1.0	1.25
0.15 (F)	0.5 (H)	0.35	0.75	1.25
0.25 (C)	0.5 (H)	0.25	0.5	1.25
0.20 (C)	0.5 (H)	0.3	0.4	1.25
0.15 (C)	0.5 (H)	0.35	0.3	1.25
0.10 (C)	0.5 (H)	0.4	0.2	1.25

iii) Stability

All the alkaloid solutions were stored at 5°C for upto three weeks.

[MLA (1) is stable under neutral conditions, and therefore at physiological pH, but has been found to be slightly unstable at low pHs. Under alkaline conditions MLA (1) has also been found to be unstable, with the succinimide ring opening at pH 9, and hydrolysis of the ester bond at pH 12 (Majak, 1993)].

iv) UV Absorption of MLA

Determination of ultraviolet (UV) absorption for MLA (1) was carried out in absolute alcohol, revealing maxima at 229 and 271nm (ε 15,350 and 3550, respectively).

c) Sample Introduction

A syringe loading sample injector was used to introduce samples onto the column in as narrow a band as possible, without any sample loss. The filling of sample loop was accomplished with a microsyringe (two times loop volume) through the needle port built into the valve shaft, thus displacing an equal volume of mobile phase.

3.3.1.2 Equipment

HPLC was carried out using a JASCO PU980 pump, a 100µl Rheodyne injection loop and a JASCO UV975 variable UV spectrophotometric detector at 270nm. A 25cm x 4.6mm i. d. Hypersil ODS 5µm column was used. All mobile phase mixtures were pumped at a flow rate of 1.0$cm^3.min^{-1}$. Chart speed was maintained at 1.0$cm.min^{-1}$using a BBC Goerz Metrawatt SE120 chart recorder. pH readings were obtained using a Kent EIL 7020 pH meter. Ultrasonic vibrations were generated using a Decon FS 100b ultrasonic bath. Determination of UV absorption was carried out using a Perkin-Elmer Lambda 3 UV/VIS spectrometer. A Finnigan 4600 mass spectrometer was used for the LC/MS work, the specifications for which are given in Section 3.3.6.

3.3.2 Optimization of the Mobile Phase

3.3.2.1 Variation in Organic Modifier Content

Chromatograms were obtained on injecting solutions containing MLA (1) ($10\mu g.cm^{-3}$) and lappaconitine (39) ($25\mu g.cm^{-3}$) in mobile phase, in duplicate, and varying the acetonitrile content of the mobile phase at a constant pH of 4.0 [**Table 9**, Figure (26) and Figure (27)]. [See Section 3.2.4]

Table 9

Optimization of the Mobile Phase: Effect of Variation of Acetonitrile Content

% CH_3CN (v/v)	t_R (mm)		κ'		$w_{1/2}$ (mm)		A_S	
	MLA	Lapp-aconitine	MLA	Lapp-aconitine	MLA	Lapp-aconitine	MLA	Lapp-aconitine
25	200	112	9.5	4.9	8	7	3.0	2.5
30	128	75.5	6.1	3.2	6	3.5	1.4	1.3
35	88	51.5	3.8	1.7	3.5	3	1.3	1.2
40	61.5	38.5	2.5	1.2	3	3	1.2	1.0

[See Section 3.2.4 for Definitions and Equations]

3.3.2.2 pH Profile

Chromatograms were obtained on injecting solutions containing MLA (1) ($10\mu g.cm^{-3}$) and lappaconitine (39) ($25\mu g.cm^{-3}$) in mobile phase, in duplicate, and varying the pH of the aqueous component of the mobile phase at a constant acetonitrile content of 35% (v/v) [**Table 10**, Figure (29) and Figure (30)]. [See Section 3.2.4]

Table 10

Optimization of the Mobile Phase: Effect of Variation of pH

Buffer pH	t_R (mm)		κ'		$w_{1/2}$ (mm)		A_S	
	MLA	Lapp-aconitine	MLA	Lapp-aconitine	MLA	Lapp-aconitine	MLA	Lapp-aconitine
3.0	39	25	2.0	0.9	2	2	1.0	1.0
4.0	91	51.5	3.8	1.7	3.5	3	1.3	1.2
5.0	147	84	6.0	3.0	8	6	1.7	1.4

[See Section 3.2.4 for Definitions and Equations]

3.3.2.3 Details of the Optimum System

Optimization of the mobile phase (buffer pH and organic modifier content), led to the use of 0.2M aqueous ammonium acetate solution adjusted to pH 5.0 with formic acid-acetonitrile (70:30 v/v). Chromatograms were obtained on injecting of a solution containing MLA (1) (10μg.cm^{-3}) and lappaconitine (39) (25μg.cm^{-3}) in mobile phase, in duplicate [**Table 11** and Figure (32)].

[See Section 3.2.4.4]

Table 11

The Optimum System

	MLA (1)		Lappaconitine (39)	
	1	2	1	2
t_R (mm)	140	150	79	84
t_O (mm)	17.5	19		
κ'	7.0	6.9	3.5	3.4
$w_{1/2}$ (mm)	7	7	5.5	4.5
w (mm)	14	13.5	11	10.5
A_S	1.3	1.15	1.2	1.1
Peak Height (mm)	37	39	172	183.5
Peak Height Ratio	0.21	0.21		
R_S	4.88	5.74		

[See Section 3.2.4 for Definitions and Equations]

3.3.3 Construction of a Calibration Curve

Chromatograms were obtained on injecting a series of solutions containing MLA (1) ($2.0\mu g.cm^{-3}$-$100\mu g.cm^{-3}$) and lappaconitine (39) (internal standard) ($25\mu g.cm^{-3}$) in mobile phase and a second series of solutions containing MLA (1) ($0.2\mu g.cm^{-3}$-$3.0\mu g.cm^{-3}$) and lappaconitine (39) ($1.25\mu g.cm^{-3}$) in mobile phase (**Tables 12a** and **12b**, respectively). These solutions were injected in duplicate (or triplicate) and using a mobile phase of 0.2M aqueous ammonium acetate solution (pH 5.0)-acetonitrile (70:30 v/v). Calibration curves were constructed for the two series, by plotting MLA peak height over lappaconitine peak height against MLA concentration. The straight lines for both series of points were fitted by linear regression equations. [See Section 3.2.5]

Table 12a

Construction of a Calibration Curve

MLA Concentration ($\mu g.cm^{-3}$)		MLA Peak Height (mm)	Lappaconitine Peak Height (mm)	Peak Height Ratio
100	1	173.5	102	1.70
	2	219	129	1.70
80	1	123.5	90	1.37
	2	122	90	1.36
60	1	119	115	1.03
	2	121	123	0.98
	3	185.5	179	1.04
40	1	87	123	0.71
	2	81	115	0.71
30	1	83	146	0.56
	2	81	144.5	0.56

Table 12a cont.

MLA Concentration ($\mu g.cm^{-3}$)		MLA Peak Height (mm)	Lappaconitine Peak Height (mm)	Peak Height Ratio
20	1	55	177	0.31
	2	60	177.5	0.34
	3	69	183	0.38
15	1	52	173.5	0.30
	2	52	181	0.29
12.5	1	40	158	0.25
	2	43	166	0.26
10	1	37	172	0.22
	2	39	183.5	0.21
8.0	1	28	196	0.14
	2	26	192	0.13
	3	27	200	0.13
6.0	1	28	169	0.17
	2	22	174	0.13
	3	20	176	0.11
4.0	1	11	125	0.09
	2	12.5	131.5	0.09
	3	12.5	132	0.09
3.0	1	11	185	0.06
	2	12	186.5	0.06
2.0	1	8.5	162	0.05
	2	8.5	164.5	0.05

Table 12b

Construction of a Calibration Curve

MLA Concentration ($\mu g.cm^{-3}$)		MLA Peak Height (mm)	Lappaconitine Peak Height (mm)	Peak Height Ratio
3.0	1	101	76.5	1.32
	2	94.5	69	1.37
2.5	1	89	88	1.01
	2	77	74	1.04
	3	68.5	77	0.89
2.25	1	90.5	102	0.89
	2	76.5	88	0.87
2.0	1	79	100	0.79
	2	62.5	89	0.70
1.75	1	39.5	75.5	0.52
	2	40	83	0.48
1.5	1	28	60	0.47
	2	37.5	66	0.57
	3	33.5	74	0.45
1.25	1	25	49	0.51
	2	28.5	57.5	0.50
1.0	1	27	81	0.33
	2	30.5	74	0.41
	3	34	77	0.44
0.75	1	19	54.5	0.35
	2	15	67	0.22
	3	14	45	0.31

Table 12b cont.

MLA Concentration ($\mu g.cm^{-3}$)		MLA Peak Height (mm)	Lappaconitine Peak Height (mm)	Peak Height Ratio
0.5	1	14	73.5	0.19
	2	18	82	0.22
	3	17	71	0.24
0.4	1	11	37.5	0.29
	2	8	40	0.20
0.3	1	11	33	0.33
	2	5	66	0.07
0.2	1	6	32	0.19
	2	12	44	0.27
	3	5	33	0.15

3.3.4 Determination of the Relative Standard Deviation

Chromatograms were obtained on injecting six replicates of solutions containing MLA (1) ($5.0\mu g.cm^{-3}$ and $25\mu g.cm^{-3}$) in mobile phase and using a mobile phase of 0.2M aqueous ammonium acetate solution (pH 5.0)-acetonitrile (70:30 v/v) **(Table 13).** [See Section 3.2.6]

Table 13

Injection of Replicate Solutions for Determination of Relative Standard Deviation

Injection Number	Peak Height (mm)	
	MLA (5.0μg.cm^{-3}) (F)	MLA (25μg.cm^{-3}) (E)
1	13	96.75
2	14	90.25
3	13.5	87.5
4	13.5	94
5	13	96
6	15	95.5

3.3.5 Assays for MLA Content in Isolated Alkaloid Samples

For the assay of MLA (1) in a crude alkaloid extract from Garden Hybrid *Delphinium* seeds, approximately 1mg of the extract, accurately weighed, was dissolved in mobile phase (10cm^3). Aliquots (1cm^3) were taken and mixed with 1cm^3 lappaconitine (39) (50μg.cm^{-3}) in mobile phase.

For the assay of MLA (1) in purified alkaloid samples, approximately 5-9mg of the alkaloid, accurately weighed, were dissolved in mobile phase (1cm^3) and mixed with 1cm^3 lappaconitine (39) [either Solution G (50μg.cm^{-3}) or Solution H (2.5μg.cm^{-3})] in mobile phase.

Chromatograms were obtained on injecting these solutions, in duplicate, and using a mobile phase of 0.2M aqueous ammonium acetate solution (pH 5.0)-acetonitrile (70:30 v/v) [**Table 14**, Figure (35) and Figure (36)]. MLA peak height over lappaconitine peak height ratios were calculated and MLA concentrations read from the calibration curve. [See Section 3.2.7]

Table 14
Analysis of Alkaloid Samples

Alkaloid		Mass of original alkaloid (mg)	Concn of alkaloid injected ($\mu g.cm^{-3}$)	Concn of lapp-aconitine injected ($\mu g.cm^{-3}$)	MLA Peak Height (mm)	Lapp-aconitine Peak Height (mm)	Peak Height Ratio
Crude Alkaloidal Material	1	1.2	60	25	92	156	0.59
	2				99	167	0.59
Lycoctonine (2)	1	5.2	2600	1.25	-	65	-
	2				-	73	-
Delpheline (219)	1	8.8	4400	25	26	111	0.23
	2				24.5	107	0.23
Neoline (246)	1	6.0	3000	1.25	-	62	-
	2				-	55	-

3.3.6 LC/MS Investigations

3.3.6.1 LC/MS Conditions

The detector was a Finnigan 4600 mass spectrometer with positive ion thermospray ionization, operating under the following conditions: Thermospray conditions; Jet 200°C, Vapour 140°C, Repeller 100volts. MS conditions; Scan Range 170-750a.m.u. in 2secs, Detector EM 1600volts, Manifold 60°C, High vacuum $4.4x10^{-5}$torr, Ionizer fore pressure 0.45torr.

3.3.6.2 LC/MS of Alkaloid Samples

For the analysis of a crude alkaloid extract from Garden Hybrid *Delphinium* seeds, approximately 2mg of the extract, accurately weighed, was dissolved in mobile phase ($20cm^3$). Likewise, for the analysis of purified alkaloid samples, approximately 1mg of the alkaloid, accurately weighed, was dissolved in mobile phase ($10cm^3$).

Chromatograms and the associated mass spectra were obtained on injecting these solutions, using a mobile phase of 0.2M aqueous ammonium acetate solution (pH 5.0)-acetonitrile (70:30 v/v) [**Table 6**, Figures (37), (38), (39), and (40)]. Structural information was obtained from the molecular ion (and ion fragments) observed for each of the alkaloid samples (**Table 7**).

[See Section 3.2.8]

CHAPTER 4

SYNTHESIS OF SMALL MOLECULE MLA ANALOGUES

4.1 AIMS

The aims of these synthetic investigations were:

i) to ascertain the minimum structural features of the nAChR antagonist MLA (1) required for activity and to explore the roles of the aromatic ester functional group of this toxic norditerpenoid alkaloid.

ii) to design a series of small molecule analogues, to contain the unusual substituted anthranilate moiety found in MLA (1).

iii) to prepare acylated cholines and homocholines from isatoic anhydride (248) and to incorporate the succinimide ring found in MLA (1), by fusion with (*RS*)-methylsuccinimide anhydride (257).

iv) to synthesize AE-bicyclic analogues of MLA (1) which contain the piperidine (E) and cyclohexane (A) rings (substituted 2-succinimidobenzoate-3-aza-bicyclo[3.3.1]nonanes).

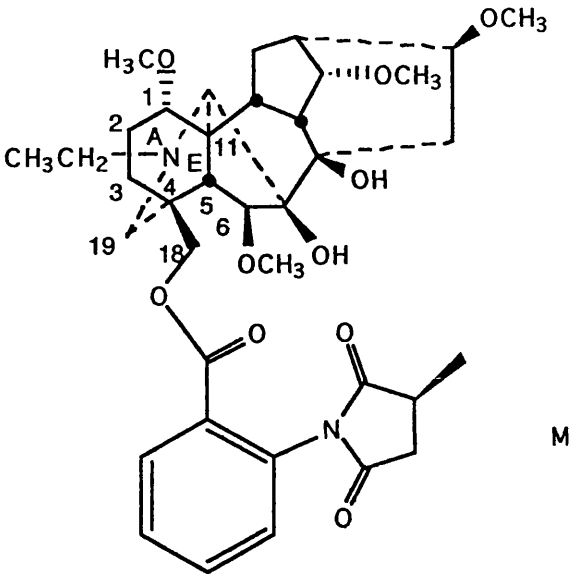
4.2 RESULTS AND DISCUSSION

4.2.1 General Strategy

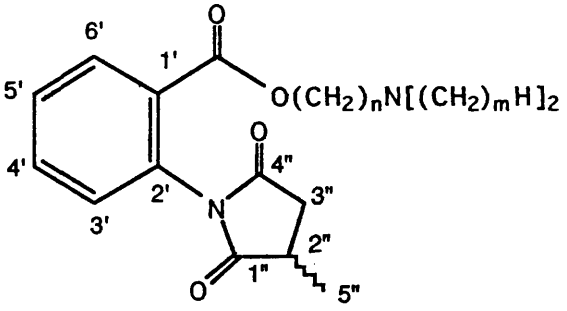
4.2.1.1 Synthetic Targets

MLA (1) is the 2-[2(*S*)-methylsuccinimido]-benzoate ester of the norditerpenoid alkaloid lycoctonine (2) (Manske, 1938). Lycoctonine (2), the parent neopentyl-like alcohol, exhibits markedly less nicotinic activity. It was rationalized that this striking difference in biological activity, must be, in part, related to the presence of MLA's *N*-succinyl anthranilate ester moiety (Jennings *et al.*, 1986 and Ward *et al.*, 1990). To test the hypothesis that this is the essential pharmacophore of the potent, competitive, and selective nicotinic receptor antagonist MLA (1), 2-(*RS*)-methylsuccinimidobenzoate esters lacking the norditerpenoid skeleton were designed.

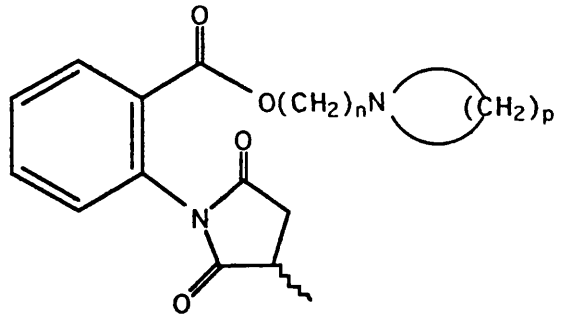
The portion of the MLA (1) molecule leading from the ester carbonyl function through C(18), C(4) and C(19) to the *N*-ethyl group bears a formal resemblance to a homocholine unit (3-aminopropan-1-ol). In our early molecular modelling studies, this motif was compared to the ester in acetylcholine, and it is proposed that it is held by the norditerpenoid skeleton such that the distance between the nitrogen and the carbonyl oxygen, is more similar to a separation of only CH_2CH_2 as found in choline. It is proposed that the competitive antagonism of nAChR displayed by MLA (1) is due to the slight distortion of the choline-like moiety into the homocholine motif found across the piperidine ring. Hence, a series of analogues [(258), (261), (264) and (267)] was proposed featuring both acylated cholines and homocholines and these esters of anthranilic acid are, in essence, acyclic analogues of the hexacyclic *N*-ethylated norditerpenoid alkaloid MLA (1).



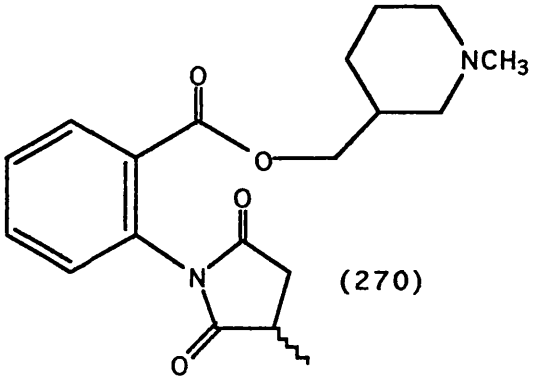
MLA (1)



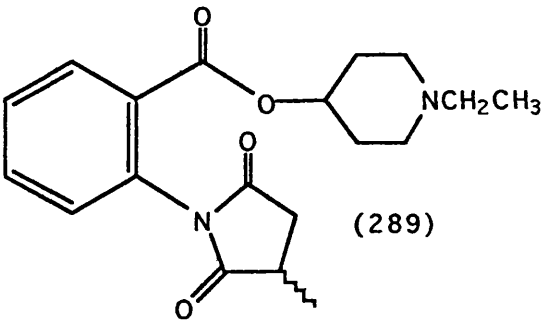
For (258), (261), (264) and (267)
n = 2,3 and m = 1,2



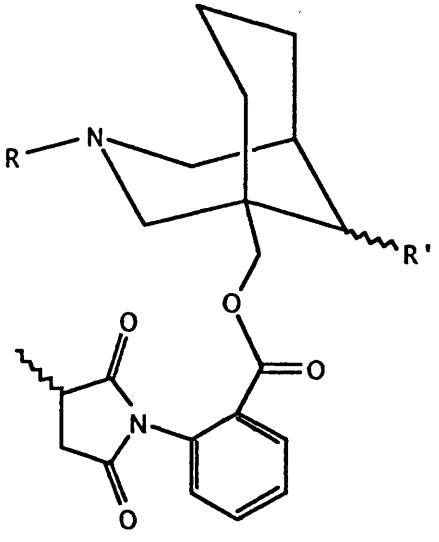
For (275), (279), (283) and (286)
n = 2,3 and p = 4,5



(270)



(289)



For (299), (300), (307), (313) and (318)
R = CH_3CH_2 or $CH_3OCH_2CH_2$
R' = OCH_3 or CH_2OCH_3

1-Methyl-3-(*RS*)-piperidinomethyl [2-(*RS*)-methylsuccinimido]benzoate (270) can be seen to possess a 3-piperidinomethyl moiety in the same manner as MLA (that is, ring E is attached to the aromatic ester portion). Compounds (275), (279), (283) and (286) with cyclic groups (that is, piperidino and pyrrolidino) at the nitrogen were also considered to be interesting target analogues. In addition, 1-ethyl-4-piperidino [2-(*RS*)-methyl succinimido]benzoate (289) was designed as a homocholine ester with restricted 'cis' conformations, as opposed to the flexible 'trans' analogues thus far in the series.

To test further the significance of the acylating anthranilic acid moiety and homocholine motif found in the proposed MLA (1) pharmacophore, synthetic routes to [3.3.1]bicyclic analogues [that is, mimicking ring E (piperidine) and ring A (cyclohexane)], functionalized as their 2-methylsuccinimidobenzoate esters, were designed. Substituted 3-aza-bicyclo[3.3.1]nonane derivatives allowed access to a series of small molecule [3.3.1]bicyclic analogues of structurally complex alkaloid MLA (1), which incorporate the homocholine motif and the *N*-ethylpiperidine moiety, but exclude oxygenation from C(1) (norditerpenoid numbering). In one series of AE-bicyclic analogues, it was decided to replace carbon C-6 (norditerpenoid numbering) with an *O*-methyl ether at C-5 [(299), (300) and (307)]. In a second series, 1-demethoxy-5-methoxymethyl analogues were proposed by extending from the ketone at C-5 to C-6 (norditerpenoid numbering) in the [3.3.1]bicycle carbon skeleton [(313) and (318)].

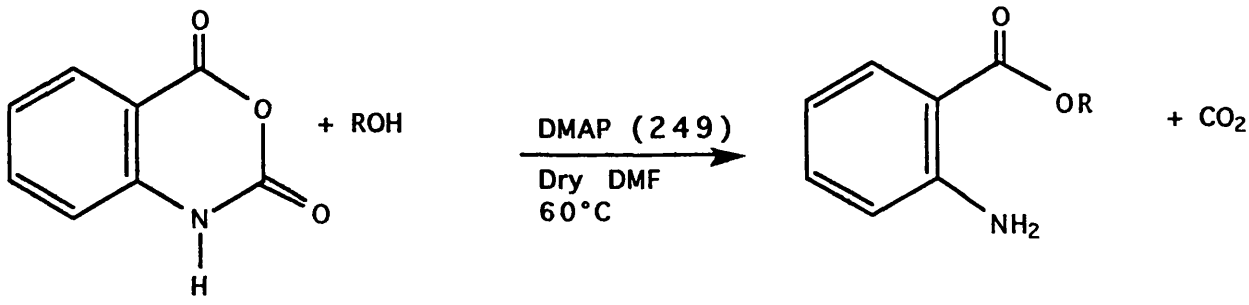
AE-bicycles of MLA (1), similar to that found in atisine (67) (Ihara *et al.*, 1990a), cardiopetaline (193) (Shishido *et al.*, 1986), and related *Aconitum* and *Delphinium* norditerpenoid alkaloids (Shimizu *et al.*, 1963) have been the subject of several synthetic investigations as described in Chapter 1. Benn

and Jacyno first reported studies into the biological activity of the parent mono- and bicyclic *N*-ethylated piperidines (Benn and Jacyno, 1983). Other workers in this area recently reporting analogues of these complex, hexacyclic norditerpenoid alkaloids include the research groups of: Whiting (Baillie *et al.*, 1994), Skăric (Skăric *et al.*, 1980), and Kraus (Kraus *et al.*, 1993). There is, however, no literature precedent for the incorporation of the unusual aromatic ester moiety.

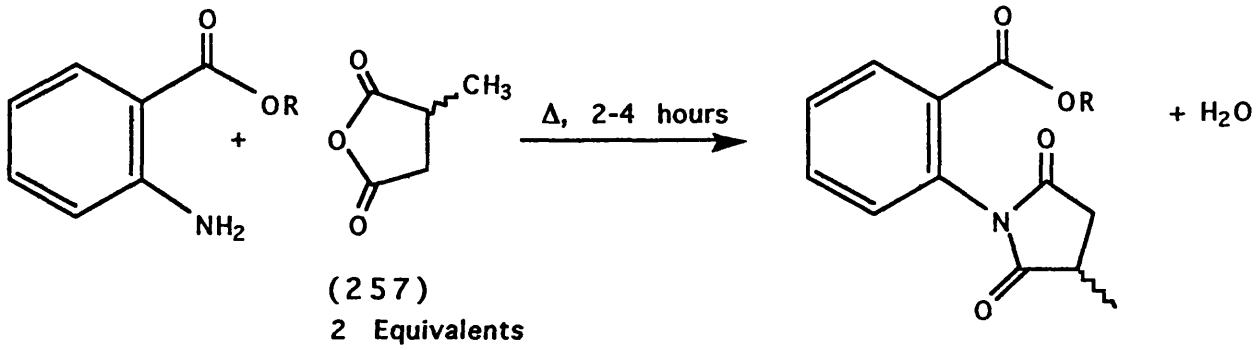
4.2.1.2 Synthetic Routes

The strategy used to prepare the desired 2-methylsuccinimidobenzoate analogues was *via* the corresponding 2-aminobenzoate compounds, as seen in Chapter 2 with regards to the conversion of inuline (40) into semi-synthetic MLA (250). The 2-aminobenzoate (anthranilate) esters can be prepared by heating the appropriate alcohol with isatoic anhydride (248) in the presence of a basic catalyst (Staiger and Miller, 1959). The subsequent conversion of the 2-aminobenzoate esters into our target compounds, incorporating imide moieties similar to the one observed in MLA (1), was achievable by fusion with two equivalents of 2-(*RS*)-methylsuccinic anhydride (257) (Morrell, 1914). Further details of this two step conversion are given in Sections 4.2.2.1 and 4.2.2.2 and an example is from the monocyclic *N*-methylated piperidine (268) to (270), *via* (269) (See Sections 4.3.2.9 and 4.3.2.10).

For analogues (275), (279), (283), (286) and all the bicyclic targets, it was necessary to synthesize the alcohols for conversion to anthranilate ester. Thus, the *N*-pyrrolidino and *N*-piperidino alcohols (273), (277), (281), and (284) were prepared following the procedure described by Leonard and Musker (1960) for tertiary aminoalcohols of this type, from the chloro-substituted alcohols (See Sections 4.3.2.11, 4.3.2.14, 4.3.1.17 and 4.3.2.20).

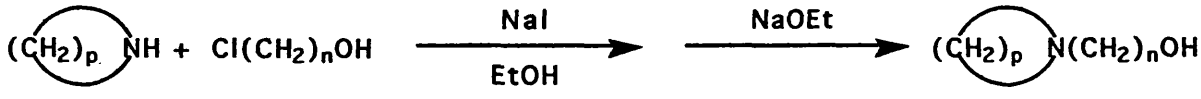


(248)

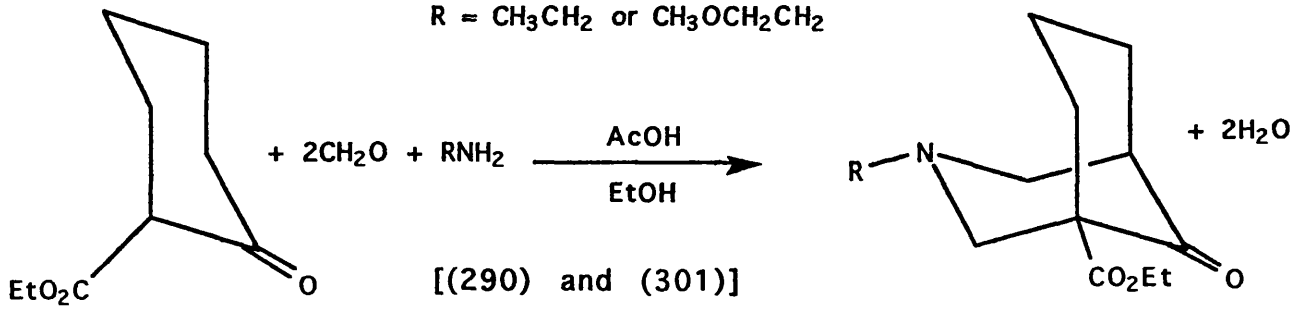


(257)
2 Equivalents

For (273), (277), (281) and (284)
n = 2,3 and p = 4,5



For (292) and (302)
R = CH_3CH_2 or $CH_3OCH_2CH_2$



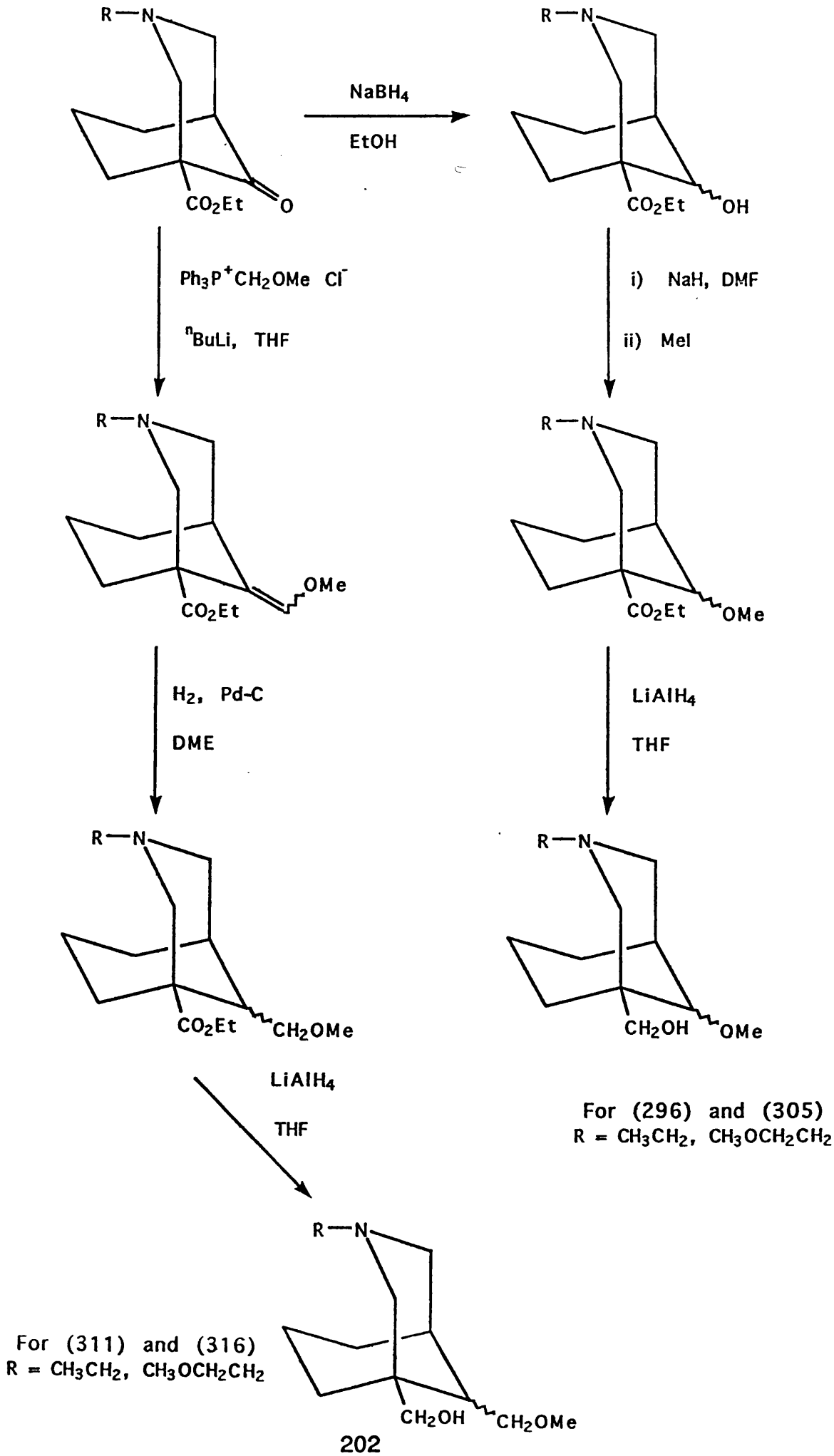
(291)

[(290) and (301)]

It was immediately apparent that a 3-aza-bicyclo[3.3.1]nonan-9-one ring system can be prepared from β-keto ester (291) which has the correct functionality, appropriately substituted, for the necessary subsequent manipulations. Thus, a double Mannich condensation between β-keto ester (291), a primary amine ($R'NH_2$), and aqueous formaldehyde was used to construct the bicyclic ring system (292)/(302) (See Sections 4.2.2.3, 4.3.2.25 and 4.3.2.33).

Neopentyl-type alcohols (296) and (305), with *O*-methyl ether at C-5 were prepared from (292) and (302), respectively in three steps; reduction of (292)/(302) with sodium borohydride (carried out at room temperature in anhydrous ethanol) to give a mixture of epimeric alcohols [9-(*RS*)-hydroxy compounds (293)/(303)], in 75-80% yields; *O*-methylation to give (294), (295)/(304); reduction of the ester functionality using lithium aluminium hydride in anhydrous tetrahydrofuran to give, in almost quantitative yields, the desired corresponding alcohols. Thus, the carboxylic ester carbonyl carbon of (292)/(302) becomes the neopentyl-type alcohol, equivalent to C(18) of lycoctonine (2), of the [3.3.1]bicycle. It is therefore intrinsic to the design of these MLA analogues, not merely a β-keto ester activating group which might have later have been removed by decarboxylation.

In the 5-methoxymethyl series of analogues, (311) and (316) were prepared, for the subsequent conversion into the corresponding anthranilate esters [(313) and (318)], from (292) and (302), respectively, again in three steps. A Wittig reaction was employed (alternatively, a Grignard-type reaction could have used) to synthesize the *E* and *Z* enol ethers (309)/(314), by preparing the phosphorus ylide *in situ* from *n*-butyllithium and (methoxymethyl)triphenyl phosphonium chloride in anhydrous tetrahydrofuran. Then catalytic hydrogenation (5 atmospheres) was used to reduce the mixture of enol ethers


R—N
CO2Et
O
NaBH4
EtOH
OH
Ph3P+CH2OMe Cl-
nBuLi, THF
i) NaH, DMF
ii) MeI
OMe
H2, Pd-C
DME
LiAlH4
THF
CH2OMe
CH2OH
For (296) and (305)
R = CH3CH2, CH3OCH2CH2
For (311) and (316)
R = CH3CH2, CH3OCH2CH2

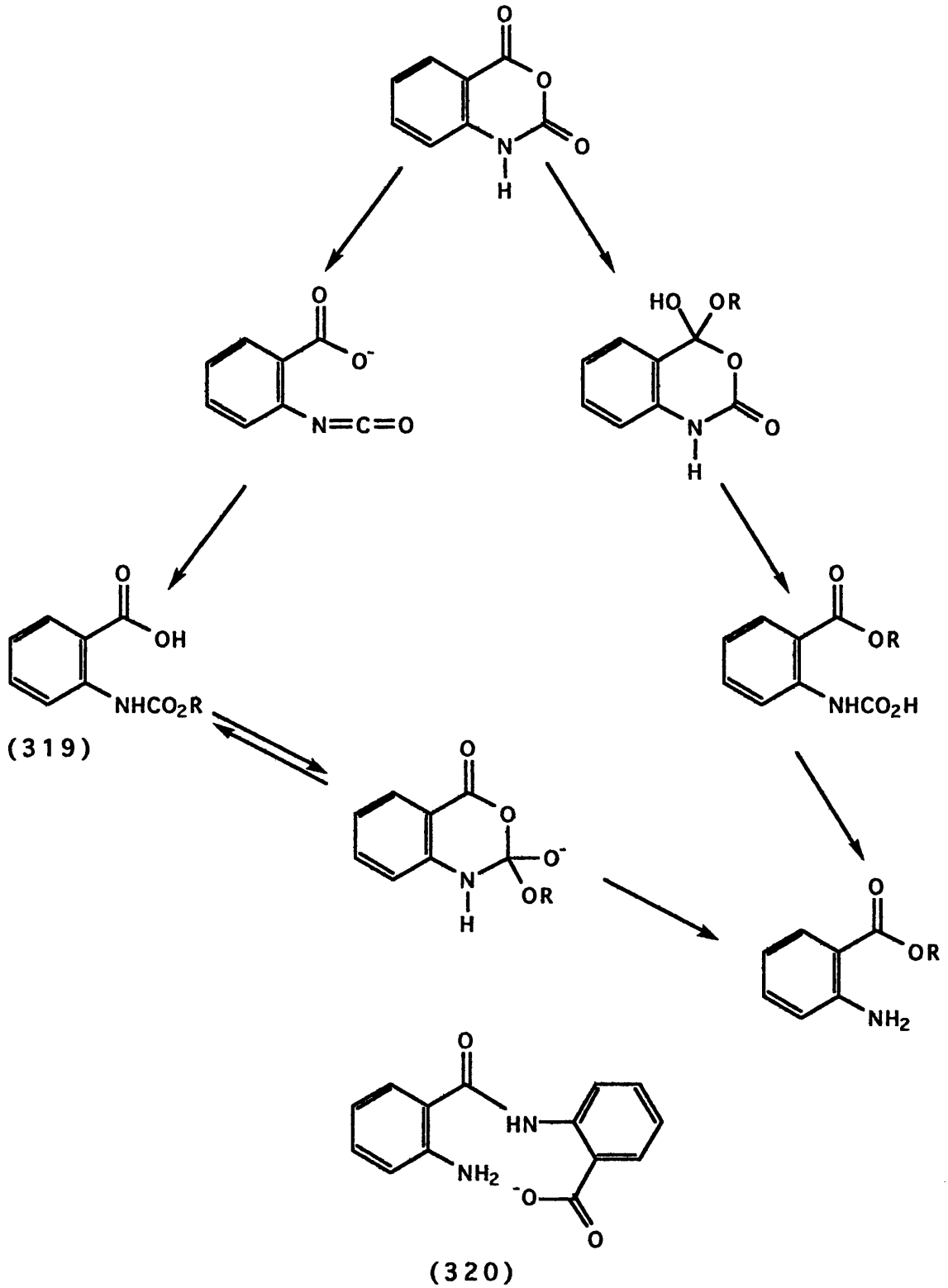
(309)/(314) to the 9-(*RS*)-methoxymethyl ethers (310)/(315) using 10% palladium on charcoal in anhydrous 1,2-dimethoxyethane, typical yields 35-45%. Finally, the carboxylate function was reduced using lithium aluminium hydride, as before.

Thus, the synthesis of these 9-substituted bicyclic alcohols [3-alkyl-3-aza-9-(*RS*)-methoxy- and methoxymethyl-bicyclo[3.3.1]nonane-1-methanols] (296), (305), (311) and (316), designed to contain the AE-ring system found in MLA (1), was achieved, in each case, in four steps from ethyl 2-cyclohexanone-1-carboxylate (291) (See Sections 4.3.2.25-4.3.2.47).

4.2.2 Major Reactions

4.2.2.1 Reactions with Isatoic Anhydride

Staiger and Miller (1959) reported the reaction of isatoic anhydride (248) with alcohols and other nucleophiles. All the anthranilate esters in our study were, therefore, prepared by heating isatoic anhydride (248) with our aliphatic acyclic, monocyclic and bicyclic alcohols in *N,N*-dimethylformamide. Anthranilate formation was accompanied by evolution of carbon dioxide gas, as expected. 4-(*N,N*-dimethylamino)pyridine (DMAP) (249) was utilized as a basic catalyst in the preparation of the anthranilates and under these conditions, the formation of the ester over the *N-o*-(carboxyphenyl) carbamate (isatoate) (319) is promoted (See scheme). In the absence of a catalyst such as this, the ureide or isatoate is expected. A number of different catalyst-solvent combinations have been reported (Staiger and Miller, 1959) but in our hands, DMAP-DMF gave satisfactory results. The nature and activity of the nucleophilic reagent, concentration and steric hindrance also determine the reaction path and the products of this ring opening. In 1991, Allen and Kirby, reported on the difference in basicity between the *E* and *Z* lone pairs of carboxylate oxygen which is consistent with our observations. Completely anhydrous conditions and freshly distilled alcohols were, therefore, were necessary in these reactions, in order to prevent the formation of dimeric species, anthraniloylanthanilic acid or salts of this acid (320) as a result of a secondary reaction (Staiger and Miller, 1959). The reaction temperature may also have promoted anthranilate formation, because isatoates, of which there was no trace (on examination of IR and ^{13}C NMR spectra), are known to rearrange to anthranilates under similar conditions (Heyman, 1978). Secondary aliphatic alcohols can be reluctant to react to form 2-amino benzoate products but 1-ethyl-4-piperidinol (287) gave


O
O
N
H
O
O⁻
N=C=O
HO
OR
O
N
H
O
OH
NHCO₂R
(319)
O
OR
NHCO₂H
O
O
O⁻
N
H
OR
O
OR
NH₂
O
HN
NH₂
⁻O
O

(320)

the required product. The yield for this reaction was low however (33%, See Section 4.3.2.23), probably as a result of isatoate formation and isatoic anhydride itself (248) competing with the bulkier (more branched close to the hydroxyl group) alcohol to react with itself.

4.2.2.2 Reactions with Methylsuccinic Anhydride

The conversion of the 2-aminobenzoate esters into their corresponding 2-methylsuccinimidobenzoate analogues, designed to incorporate imide moieties similar to the one observed in MLA (1), was achieved by fusion with two equivalents of 2-(*RS*)-methylsuccinic anhydride (257) (Morrell, 1914). Typically the two reactants were heated together for 2-4 hours, resulting in a hard gum, which on purification yielded of 40-70% of the desired product.

There are a number of literature methods for preparing imides, and even aryl imides, but not aryl succinimides. For example, the preparation of aryl maleimides from maleic anhydride and both substituted and unsubstituted aromatic amines is described by Cava *et al.* (1961) and Augustin and Müller (1985), mostly involving dehydrative cyclization of the intermediate maleic acid monoamide. The preparation of cyclic imides has been well documented; in 1984, Flitsch and Ruβkamp described the synthesis of succinimides from bromo and nitro substituted aromatic compounds; in 1914, Morrell reacted aniline with succinic and methylsuccinic acid; in 1991, Garner *et al.*, 1991 prepared maleiimides by condensation of maleic anhydride with benzylamines, using acetic anhydride mediated dehydration to bring about the ring closure; Balasubramaniyan and Argade (1987 and 1988) also used acetic anhydride to dehydrate carboxymaleanilic acids; Hoffmann and Schiff-Shenhav (1962), Greenstein and Winitz (1961), Bose *et al.* (1958) and Kidd and King (1948) investigated the reaction of amino acids with phthalic anhydride, under a variety of reaction conditions, including fusing, to give phthaloylamino acids and phthalimides. In 1991, Ito *et al.*, 1991 disclosed the possible problems associated with dehydration reactions involving maleimides.

4.2.2.3 Double Mannich Reactions

A double Mannich condensation between β-keto ester (291), a primary amine [ethylamine (290) or β-methoxyethylamine (301)], and aqueous formaldehyde (37%, 2 equivalents, reflux in ethanol in the presence of a catalytic amount of glacial acetic acid) was used provide rapid and efficient entry (typical yields 50-70%) to the 3-aza-bicyclo[3.3.1]nonane ring system (292)/(302), and thus to the substituted bicyclic derivatives. The procedure used was based on that utilized by Blicke and McCarty (1959) for disubstituted cyclohexanones such as 2,6-dimethyl ester. Likewise, Schneider and Götz (1961) used a Mannich reaction to prepare the naturally occurring alkaloid, pseudopelletierin, 3-methyl-3-azabicyclo[3.3.1]nonan-9-one. The chief applications of the Mannich reaction, useful in the preparation of ketonic amines, involves condensation of formaldehyde and a secondary amine hydrochloride with a ketone having an active hydrogen atom.

Both (290) and (301) were used as the amine to give two parallel sets of analogues (See Sections 4.3.2.25 and 4.3.2.33). The basicity of a tertiary amine can be significantly reduced [lowered by essentially a full pK_a unit (Perrin *et al.*, 1981)] by the addition of a β-methoxyethyl group. It was hoped that modulating the pK_a, would result in this series being more lipophilic at physiological pH (Perrin *et al.*, 1981).

Speckamp *et al.* (1970) reported a boat-chair form for bicyclo[3.3.1]nonanes similar to our system, but prepared using an enamine method not a Mannich reaction, and discussed briefly the ^1H NMR spectra for such compounds.

It is important to note that our monocyclic starting material, ethyl 2-cyclohexanone-1-carboxylate (291), has a chiral centre and that it exists as the mixture. Thus, all subsequent bicyclic compounds have two [for example, (292)] or more [for example, (293)] chiral centres and exist as mixtures, at positions 1 and 5, as well as position 9, where relevant (See structure for numbering).

4.2.3 Characterization of Products

4.2.3.1 Trends Observed for 2-Aminobenzoates

For the (*N,N*-dialkylamino)alkyl 2-aminobenzoates series, a trend in boiling point, that is increasing from 106-108°C/0.02mmHg [lit. Bp 119-122°C/0.2mmHg (Krapcho and Turk, 1966)] for 2-(*N,N*-dimethylamino)ethyl 2-aminobenzoate (256) to Bp 122°C/0.01mmHg for 3-(*N,N*-diethylamino)propyl 2-aminobenzoate (266 with molecular weight, was observed, as might be expected. Data for 2-(*N,N*-dimethylamino)ethyl 2-aminobenzoate (256) is available in Birmachu and Reed, 1988. Niemann and Dekker (1966) provided data for 2-(*N,N*-diethylamino)ethyl 4-aminobenzoate type compounds, namely the 3-amino derivative as well as 2-amino derivative.

(269) was purified by distillation under reduced pressure (Bp 174-176°C/0.15mmHg) and was crystallized from methanol to give a white crystalline solid with Mp 60-61°C. (278) was obtained in almost quantitative yield, while 1-ethyl-4-piperidino 2-aminobenzoate (288) (a white crystalline solid with Mp 252-254°C) was obtained in a low yield 33%, as discussed in 4.2.2.1.

NMR, MS and TLC data for the bicyclic 2-aminobenzoates {considering all four series of compounds, 3-ethyl- and 3-(2-methoxyethyl)-, 9-(*R* and *S* or *RS*)-methoxy- and 9-(*RS*)-methoxymethyl-3-aza-bicyclo[3.3.1]nonane-1-methyl 2-aminobenzoates [(297) and (298), (306), (312), and (317)]} can be compared to that of inuline (40), relative to lycoctonine (2) and MLA (1). In addition these compounds were prepared in low yields, (297) in 17%, (298) in 16% and (306) in 38%.

4.2.3.2 Trends Observed for 2-Methylsuccinimidobenzoates

It is interesting that direct fusion with two equivalents of starting anhydride, in the methylsuccinic (257) (and unsubstituted succinic) case, were required to obtain the succinimidobenzoate products and prevent isolation of the ring opened products. In order to simplify ^{1}H nmr analysis, initially succinic anhydride was chosen as the anhydride and fusion with 2-(*N,N*-dimethylamino)ethyl 2-aminobenzoate (256) was attempted. However, the reaction produced a miriad of non-polar products (TLC analysis) and interpretation of the ^{1}H nmr spectrum of the crude material was difficult. A peak at δ2.46ppm (DMSO-d_6) indicated the presence of succinic acid. Imide formation involves the generation of water which could then react with unreacted succinic anhydride to give the acid which could then result in side reactions. The reaction of the same starting ester (256) with (*RS*)-methylsuccinic anhydride (257) using the same conditions and work-up procedure gave the expected diastereomeric 2-methylsuccinimide (258). The significance of the methyl group is surprising and failure to obtain the corresponding imide, lacking this slightly electron donating group cannot be explained. The COSY spectrum of one of the other three successfully prepared imides, 2-(*N,N*-diethylamino)ethyl [2-(*RS*)-methylsuccinimido] benzoate (264), illustrating clearly the couplings observed. It is interesting to note that in the 90MHz ^{1}H nmr, as reported in the experimental section (See Section 4.3.2.2), there is some overlap of the signals for H-5' and H-4' in the aromatic region and the H-5" protons appear as a doublet. In contrast, the spectrum obtained as part of the COSY experiment, which was still carried out in $CDCl_3$ but at 400MHz, shows a d, t, t, d pattern in the aromatic region and the succinyl methyl group can be seen as a broad singlet. This observation was also made for imides (261), (264), and (267). Changes in chemical shift and loss of resolution are not uncommon on altering the field strength.

Of the acyclic and monocyclic compounds, 1-ethyl-4-piperidino [2-(*RS*)-methylsuccinimido]benzoate (289), although obtained in low yield 34%, gave the most interesting ^{1}H NMR spectrum to analyze, with the axial and equatorial protons in position 6 being indistinguishable but in positions 3 and 5 the protons come, appropriately, at slightly different chemical shifts form each other.

The NMR and MS data for MLA (1), the compound on which all the small molecule analogues are modelled, follows the same trends as for the synthetic compounds, for example the ^{1}H NMR shifts and multiplicities of the aromatic and succinimide ring protons. Chromatographically, the synthetic mimics are not of particular interest.

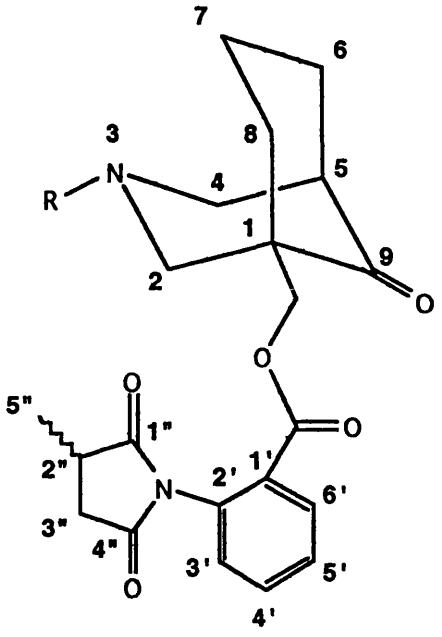
4.2.3.3 Trends Observed for Monocyclic Alcohols

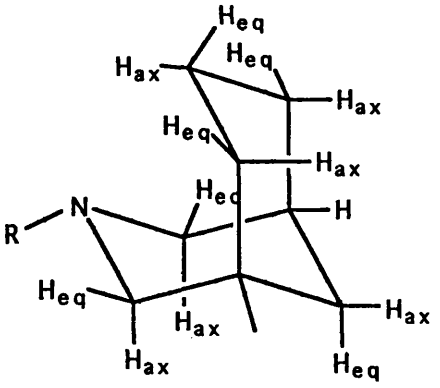
2-*N*-Pyrrolidino-1-ethanol (273), 3-*N*-Pyrrolidino-1-propanol (277), 2-*N*-Piperidino-1-ethanol (281), 3-*N*-Piperidino-1-propanol (284), were synthesized from for example, trimethylene chlorohydrin and excess amine, with sodium iodide as nucleophilic catalyst, such that the chloro substituent is displaced by an iodo group. These monocyclic alcohols were each purified by distillation under reduced pressure (~20-25mmHg); a general trend of increasing boiling point with increasing molecular weight was observed as expected (Leonard and Musker, 1960). The chemical shifts and multiplicities in the ^{1}H NMR spectra for the methylene protons in the rings and chains were explainable, such that the deshielding effect of the oxygen atom was observed to be greater than that of the nitrogen atom.

4.2.3.4 Trends Observed for Bicyclic Compounds

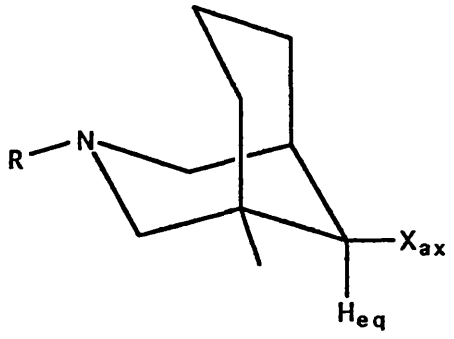
4.2.3.4.1 NMR Assignment

Assignment of ^{1}H NMR spectra of bicyclic compounds (292) to (318) was complex due to the large number of both geminal, vicinal and, *W*-couplings of spectroscopically similar protons. It was found that, at most stages in our synthetic routes, only modest separation of the epimers at C-9 was obtainable by chromatography over silica gel, which made structure elucidation for individual compounds more complicated; but *O*-methyl ethers (294), (295) [and equally (297), (298)] were separable, which allowed for full characterization following from detailed inspections of their ^{1}H NMR spectra (COSY and nOe experiments). Thus, it was found that, while unfortunately δ3.53ppm (d, J_{59} 3Hz) and δ3.31ppm (OCH_3, axial to cyclohexane) and δ3.49ppm (d, J_{59} 2Hz) and δ3.31ppm (OCH_3, equatorial to cyclohexane) were not especially diagnostic in the 1D spectra, it was possible to assign the epimers as such. This information was used, where appropriate, to assign diastereoisomers with the substituent at C-9 axial to the cyclohexane ring of the bicycle as *R* and diastereoisomers with the substituent equatorial as *S*. It was helpful to utilize the ^{1}H NMR assignments confidently made for the ketoesters (292) and (302) as a basis for subsequent members of the series of analogues, especially for the assignment of 4_{eq}-H, 4_{ax}-H, 2_{eq}-H, and 2_{ax}-H.

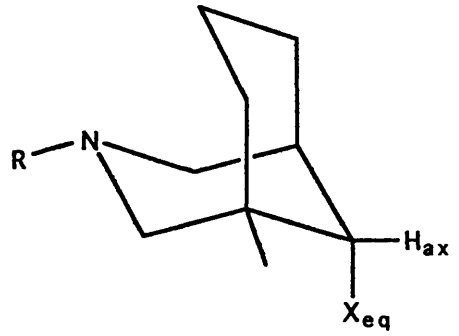




R = CH_3CH_2 or $CH_3OCH_2CH_2$

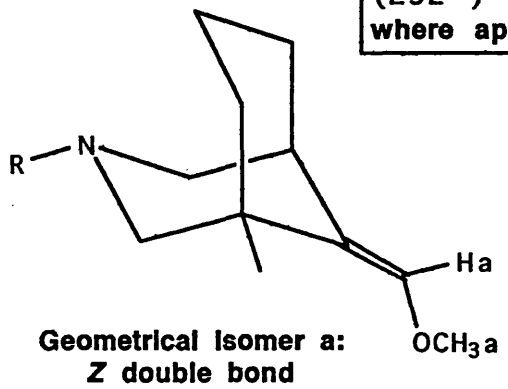


Diastereoisomer a:
Substituent X at C-9
axial to cyclohexane ring.

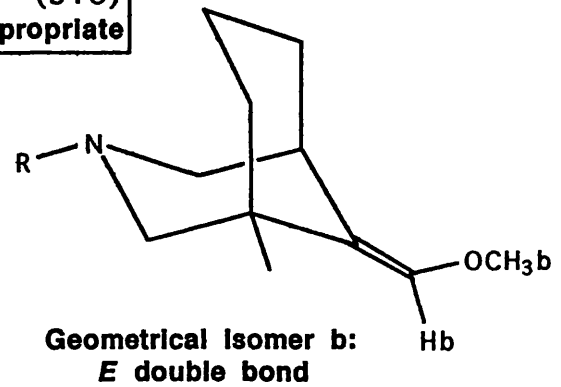


Diastereoisomer b:
Substituent X at C-9
equatorial to cyclohexane ring.

For Compounds (292) - (318) where appropriate



Geometrical isomer a:
***Z* double bond**



Geometrical isomer b:
***E* double bond**

HETCOR spectra, as well as other 1D and 2D ^{1}H NMR and ^{13}C NMR spectra, were obtained for all separable isomers and for final products [(299), (300), (307), (313), and (318)] and were found to facilitate assignment of the numerous methylene groups around the bicyclic systems.

Comparing the spectra obtained for corresponding members of the two series (N-CH_2CH_3 and N-$CH_2CH_2OCH_3$) [for example, methyl ethers (310) and (315)] revealed some interesting trends in the ^{1}H NMR and ^{13}C NMR spectra, as did the consideration of the relative electron withdrawing effects of the oxygen substituents at C-9 and C-1).

4.2.3.4.2 Effect of Substituent at Nitrogen

In the ^{1}H NMR spectra for the series of compounds starting from (292) that is, with an ethyl group at the nitrogen atom at position 3, a triplet in the region of δ1.1ppm and a quartet in the range δ2.5-2.8ppm are observed, whereas for the series of compounds with a methoxyethyl substituent at the nitrogen, culminating in (307) and (318), a downfield triplet at approximately δ1.4ppm, another triplet at δ2.7-3.0ppm plus the methoxyl signal in the region of δ3.3ppm are seen.

The nitrogen substituent (ethyl or methoxyethyl) was seen to effect the chemical shift (both ^{1}H NMR and ^{13}C NMR) of nuclei in close proximity to it, that is, at C-2 and C-4, and to a small degree at C-5, C-6, C-7, and C-8, due to the electron accepting character of the methoxyl group. This deshielding effect is almost negligible when comparing the spectra obtained for (312) and (317), for example.

4.2.3.4.3 Effect of Substituent at C-9

The nature (and orientation, where appropriate) of the substituent at C-9 (for example, ketone, hydroxyl, methoxyl, and methoxymethyl) effects the chemical shifts (both ^{1}H NMR and ^{13}C NMR) observed for nuclei in close proximity to it, that is, at C-5 and to a small degree at C-2, C-4, C-6, and C-8. In particular, NMR was found to be an excellent technique for monitoring the disappearance of starting ketoester [(292) and (302)] and appearance of product for the formation of methylidene compounds (309) and (314) and likewise for their conversion into (310) and (315), respectively; for example, the alkenyl methine proton and the methoxyl protons for geometrical isomer a of (309) (*Z* double bond) gave ^{1}H NMR signals at δ5.77ppm and δ3.47ppm and the spectra for geometrical isomer b (*E* double bond) revealed singlets at δ5.70ppm and δ3.53ppm. Infact, considerable practical problems were encountered with the Wittig reaction; initially the disappearance of the brilliant red-orange colour, produced on formation of the ylide, was believed to indicate the synthesis of the desired compound (309), but infact deterioration, rather than reaction, of the ylide was occurring and extremely pure and dry starting materials, reagents, solvent and inert gas were required for even modest yields to be obtained.

4.2.3.4.4 Effect of Substituent at C-1

The disappearance of the ^1H NMR signals for the methylene and methyl protons in the ethyl ester functionality (at approximately δ4.1-4.2ppm and δ1.3ppm, respectively) for compounds (294), (295) (304), (310), and (315) was indicative of successful lithium aluminium hydride reduction to the corresponding primary alcohols. Likewise, appearance of peaks in the aromatic regions of the ^1H NMR and ^{13}C NMR spectra obtained for products isolated from the isatoic anhydride reactions with alcohols (296), (305), (311), and (316), suggested formation of the 2-aminobenzoate esters. These conversions are analogous to that of lycoctonine (2) into inuline (40) (as discussed in Chapter 2), and thus in the same way that some comparisons can be made between the spectra for MLA (1) and our final synthetic products (the 2-succinimidobenzoate-3-aza-bicycles), parallels between these compounds can be made. In particular, the protons in 1,2-disubstituted benzene ring show similar chemical shifts and size of *ortho*, *meta*, and *para* coupling constants across the range of comparable compounds.

In addition, the nature of the substituent at C-1 (for example, ethyl carboxylate, hydroxymethyl, and CH_2OCOAr) effects the chemical shifts (both ^1H NMR and ^{13}C NMR) observed for nuclei in close proximity to it, that is, at C-9, C-2, and C-8.

4.3 EXPERIMENTAL

4.3.1 Instrumentation and Experimental Techniques

4.3.1.1 Solvents and Reagents

Solvents and reagents were dried and purified prior to use according to the procedures described in the "Purification of Laboratory Chemicals" (Perrin and Armarego, 1988). Anhydrous tetrahydrofuran and diethyl ether were obtained by distillation from sodium/benzophenone. Water refers to distilled water.

4.3.1.2 Analysis and Spectroscopy

Ultraviolet (UV) spectra were obtained in aqueous solution and recorded using a Perkin-Elmer Lambda 3 UV/VIS spectrometer as described in Section 3.3.1.

Infrared (IR) spectra were obtained using thin discs (KBr) and recorded in the range 4000-600cm^{-1} using a Perkin-Elmer 782 Infrared spectrophotometer and peaks are reported (λ_{max}) in wavenumbers (cm^{-1}). Intensities are expressed subjectively as strong (s), medium (m), and weak (w).

All other general experimental details are as described in Sections 2.3.1 or 3.3.1, unless otherwise stated.

4.3.2 Experimental Work

4.3.2.1 2-(*N,N*-Dimethylamino)ethyl 2-aminobenzoate (256) (Chemical Abstracts Registry Number [14115-02-7]).

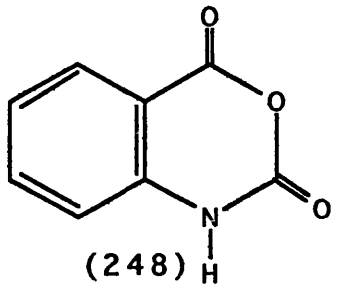
To a stirred solution of isatoic anhydride (248) (4.08g, 25.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.30g, 2.5mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (40cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 2-(*N,N*-dimethylamino)ethanol (255) (2.76cm^3, 27.5mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (17h). The mixture was cooled to 25°C (45min) and then partitioned between ethyl acetate (70cm^3) and water (70cm^3). The aqueous layer was extracted with ethyl acetate (3 x 70cm^3) and the combined organic layers were then washed successively with water (3 x 70cm^3) and brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber viscous oil was purified by distillation under reduced pressure {Bp 106-108°C/0.02mmHg [lit. Bp 119-122°C/0.2mmHg (Krapcho and Turk, 1966)]} to give the 2-aminobenzoate (256) as a colourless oil (3.27g, 63%). TLC (methanol-dichloromethane 1:9, $R_f = 0.3$); λ_{max} (EtOH)/nm: 248 (ε 9,500) and 338 (ε 6,000); υ_{max} (film)/cm^{-1}: 3490m, 3380m, 3150w, 2955s, 2830w, 2780w, 1695s, 1620s, 1595m, 1460w, 1375m, 1250s and 1110m; δ_H ($CDCl_3$)/ppm (90MHz) 7.84 (1H, dd, *J* 7.8 and 2.5, 6'-H), 7.20 (1H, td, *J* 7.8 and 2.5, 4'-H), 6.64-6.48 (2H, m, 3'- and 5'-H), 5.90 (2H, br s, NH_2), 4.35 (2H, t, *J* 6.5, 1-H_2), 2.64 (2H, t, *J* 6.5, 2-H_2) and 2.27 [6H, s, $N(CH_3)_2$]; $C_{11}H_{16}N_2O_2$ requires MW 208 and C, 63.4; H, 7.7; N, 13.4%. Found: m/z (CI, isobutane) 209 (MH^+, 95%), 72 {$[CH_2CH_2N(CH_3)_2]^+$, 100%} and C, 63.4; H, 8.0; N, 13.3%. [See Sections 4.2.2.1 and 4.2.3.1]

$HOCH_2CH_2N(CH_3)_2$ (255)

$HOCH_2CH_2CH_2N(CH_3)_2$ (259)

$HOCH_2CH_2N(CH_2CH_3)_2$ (262)

$HOCH_2CH_2CH_2N(CH_2CH_3)_2$ (265)



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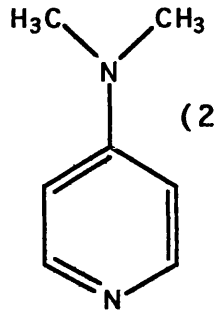

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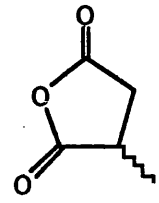

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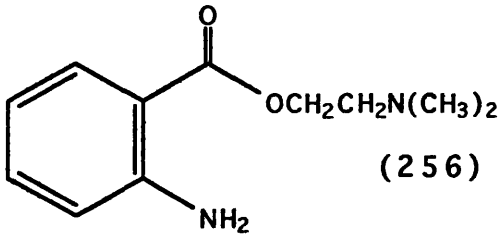

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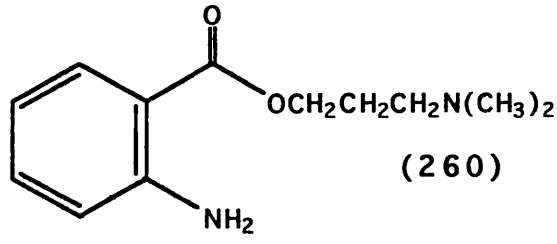

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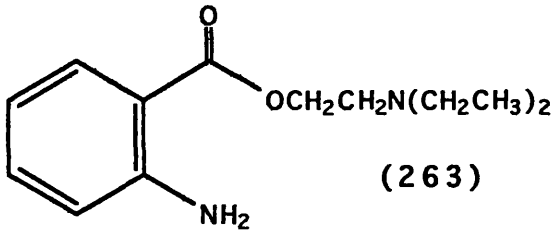

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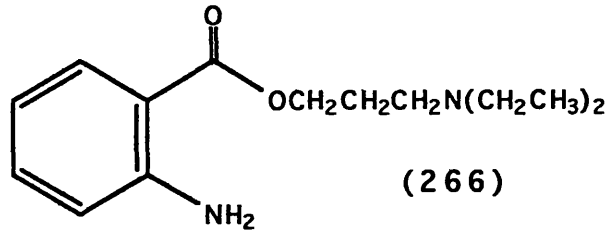

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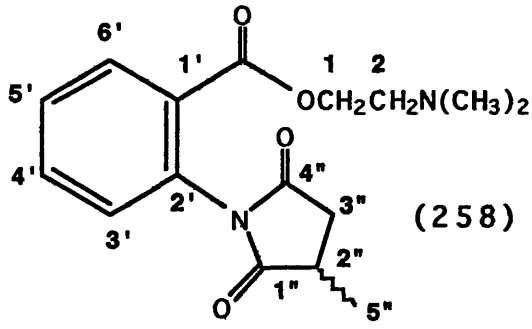

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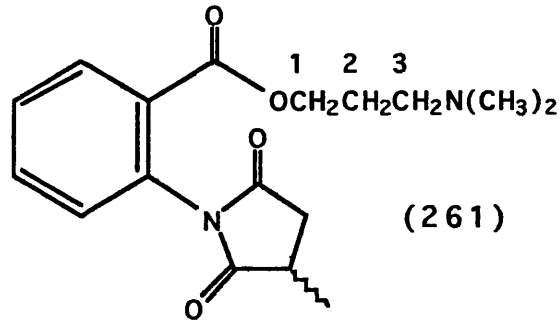

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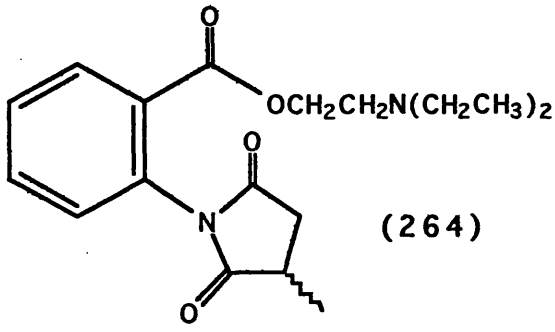

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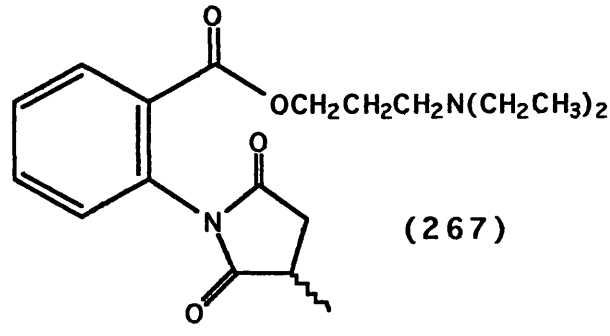


(267)

4.3.2.2 2-(*N,N*-Dimethylamino)ethyl [2-(*RS*)-methylsuccinimido] benzoate (258)

A mixture of 2-aminobenzoate (256) (1.06g, 5.0mmol) and (*RS*)-methylsuccinic anhydride (257) (0.96cm^3, 10.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (4h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (50cm^3) and water (50cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (3 x 70cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange oil was purified over silica gel (70g) eluted with 0-30% methanol in ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (258) as a pale yellow oil (0.83g, 55%). TLC (methanol-ethyl acetate 1:3, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 2980m, 2830w, 2790w, 1720s, 1610s, 1500m, 1460m, 1400s, 1265s, 1190s, 1140m and 1110w; δ_H ($CDCl_3$)/ppm (90MHz) 8.13 (1H, dd, *J* 7.6 and 2.5, 6'-H), 7.63 (1H, td, *J* 7.6 and 2.5, 4'-H), 7.51 (1H, td, *J* 7.6 and 2.5, 5'-H), 7.25 (1H, dd, *J* 7.6 and 2.5, 3'-H), 4.35 (2H, t, *J* 5.7, 1-H_2), 3.36-2.92 (3H, m, 2"-H and 3"-H_2), 2.68 (2H, t, *J* 5.7, 2-H_2), 2.32 [6H, s, $N(CH_3)_2$] and 1.52 (3H, d, *J* 6.3, 5"-H_3); δ_C ($CDCl_3$)/ppm (68MHz) 179.9 (C-1"), 175.9 (C-4"), 164.4 (OC=O), 133.4 (C-5'), 132.6 (C-2'), 131.7 (C-6'), 129.7 (C-3'), 129.3 (C-4'), 127.3 (C-1'), 62.9 (C-1), 57.4 (C-2), 45.5 [$N(CH_3)_2$], 37.0 (C-3"), 35.3 and 35.2 (C-2", both isomers) and 16.5 and 16.3 (C-5", both isomers); $C_{16}H_{20}N_2O_4$ requires MW 304 and C, 63.1; H, 6.6; N, 9.2%. Found: m/z (+)FAB 305 (MH^+, 100%), (-)FAB 58 {[$CH_2N(CH_3)_2$]$^-$, 100%} and C, 63.0; H, 6.7; N, 9.1%. [See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.3 3-(*N,N*-Dimethylamino)propyl 2-aminobenzoate (260)

To a stirred solution of isatoic anhydride (248) (4.08g, 25.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.30g, 2.5mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (40cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 3-(*N,N*-dimethylamino)propan-1-ol (259) (3.25cm^3, 27.5mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (16h). The mixture was cooled to 25°C (1h) and then partitioned between ethyl acetate (70cm^3) and water (70cm^3). The aqueous layer was extracted with ethyl acetate (3 x 70cm^3) and the combined organic layers were then washed successively with water (3 x 70cm^3) and brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber oil was purified by distillation under reduced pressure (Bp 109-111°C/0.02mmHg) to give the 2-aminobenzoate (260) as a colourless oil (3.16g, 57%). TLC (methanol-dichloromethane 1:9, R_f = 0.2); λ_{max} (EtOH)/nm: 247 (ε 18,400) and 338 (ε 12,300); υ_{max} (film)/cm^{-1}: 3490m, 3380m, 3160w, 2950s, 2830m, 2780m, 1695s, 1625s, 1465w, 1390m, 1250s and 1110m; δ_H ($CDCl_3$)/ppm (90MHz) 7.69 (1H, dd, *J* 7.9 and 2.0, 6'-H), 7.24 (1H, td, *J* 7.9 and 2.0, 4'-H), 6.81-6.41 (2H, m, 3'- and 5'-H), 5.80 (2H, br s, NH_2), 4.21 (2H, t, *J* 6.3, 1-H_2), 2.33 (2H, t, *J* 6.3, 3-H_2), 2.12 [6H, s, $N(CH_3)_2$] and 1.79 (2H, quin, *J* 6.3, 2-H_2); $C_{12}H_{18}N_2O_2$ requires MW 222 and C, 64.8; H, 8.2; N, 12.6%. Found: m/z (CI, isobutane) 223 (MH^+, 100%), 86 {$[CH_2CH_2CH_2N(CH_3)_2]^+$, 28%} and C, 64.7; H, 8.3; N, 12.7%. [See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.4 3-(*N,N*-Dimethylamino)propyl [2-(*RS*)-methylsuccinimido] benzoate (261)

A mixture of 2-aminobenzoate (260) (1.55g, 7.0mmol) and (*RS*)-methylsuccinic anhydride (257) (1.59g, 14.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (4h). The resulting dark brown gum was cooled to 25°C (30min) and then dissolved in ethyl acetate (50cm^3). This solution was extracted with water (50cm^3) and the aqueous layer concentrated to dryness under reduced pressure which afforded an orange oil. This residual oil was washed with saturated aqueous sodium hydrogen carbonate solution (3 x 70cm^3) and then extracted with ethyl acetate (3 x 70cm^3). The combined organic layers were washed with brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual yellow oil was purified over silica gel (70g) eluted with 0-30% methanol in ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (261) as a light golden oil (1.08g, 48%). TLC (methanol-ethyl acetate 1:3, $R_f = 0.2$); υ_{max} (film)/cm^{-1}: 2980m, 2830w, 2800w, 1720s, 1610w, 1500m, 1460m, 1400s, 1270s, 1190m, 1140m and 1090w; δ_H ($CDCl_3$)/ppm (90MHz) 8.10 (1H, dd, *J* 7.6 and 2.6, 6'-H), 7.63 (1H, td, *J* 7.7 and 2.6, 4'-H), 7.53 (1H, td, *J* 7.7 and 2.6, 5'-H), 7.23 (1H, dd, *J* 7.7 and 2.6, 3'-H), 4.26 (2H, t, *J* 6.4, 1-H_2), 3.20-2.90 (2H, m, 2"- and 3"-H), 2.55-2.48 (1H, m, 3"-H), 2.39 (2H, t, *J* 6.4, 3-H_2), 2.23 [6H, s, $N(CH_3)_2$], 1.88 (2H, quin, *J* 6.4, 2-H_2) and 1.45 (3H, d, *J* 6.4, 5"-H_3); δ_C ($CDCl_3$)/ppm (68MHz) 179.8 (C-1"), 175.9 (C-4"), 164.3 (OC=O), 133.2 (C-5'), 132.6 (C-2'), 131.5 (C-6'), 129.9 (C-3'), 129.2 (C-4'), 127.4 (C-1'), 63.5 (C-1), 56.0 (C-3), 45.3 [$N(CH_3)_2$], 36.9 (C-3"), 35.3 and 35.1 (C-2", both isomers), 26.7 (C-2) and 16.4 and 16.2 (C-5", both isomers); $C_{17}H_{22}N_2O_4$ requires MW 318 and C, 64.1; H, 7.0; N, 8.8%. Found: m/z (+)FAB 317 ($M-H^+$, 100%), (-)FAB 318 (M^-, 5%) and 58 {$[CH_2N(CH_3)_2]^-$, 100%} and C, 63.9; H, 7.0; N, 8.6%.

[See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.5 2-(*N,N*-Diethylamino)ethyl 2-aminobenzoate (263)
(Chemical Abstracts Registry Number [10369-93-4]).

To a stirred solution of isatoic anhydride (248) (4.08g, 25.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.30g, 2.5mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (40cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 2-(*N,N*-diethylamino)ethanol (262) (3.65cm^3, 27.5mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (17h). The mixture was cooled to 25°C (1h) and then partitioned between ethyl acetate (70cm^3) and water (70cm^3). The aqueous layer was extracted with ethyl acetate (3 x 70cm^3) and the combined organic layers were then washed successively with water (3 x 70cm^3) and brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber oil was purified by distillation under reduced pressure (Bp 136-138°C/0.1mmHg) to give the 2-aminobenzoate (263) as a colourless oil (3.82g, 65%). TLC (methanol-dichloromethane 1:9, $R_f = 0.3$); λ_{max} (EtOH)/nm: 248 (ε 9,500) and 338 (ε 6,000); υ_{max} (film)/cm^{-1}: 2980s, 2955s, 1695s, 1625s, 1595m, 1460w, 1390m, 1250s and 1110m; δ_H ($CDCl_3$)/ppm (90MHz) 7.70 (1H, dd, *J* 8.1 and 2.5, 6'-H), 7.25 (1H, td, *J* 8.1 and 2.5, 4'-H), 6.81-6.43 (2H, m, 3'- and 5'-H), 5.90 (2H, br s, NH_2), 4.24 (2H, t, *J* 5.4, 1-H_2), 2.72 (2H, t, *J* 5.4, 2-H_2), 2.53 [4H, q, *J* 7.2, $N(CH_2\ CH_3)_2$] and 0.97 [6H, t, *J* 7.2, $N(CH_2CH_3)_2$]; $C_{13}H_{20}N_2O_2$ requires MW 236 and C, 66.1; H, 8.5; N, 11.9%. Found: m/z (CI, isobutane) 237 (MH^+, 100%), 86 {$[CH_2N(CH_2CH_3)_2]^+$, 79%} and C, 65.9; H, 8.8; N, 11.9%.

[See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.6 2-(*N,N*-Diethylamino)ethyl [2-(*RS*)-methylsuccinimido] benzoate (264)

A mixture of 2-aminobenzoate (263) (1.65g, 7.0mmol) and (*RS*)-methylsuccinic anhydride (257) (1.59g, 14.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (4h). The resulting dark brown gum was cooled to 25°C (30min) and then dissolved in saturated aqueous sodium hydrogen carbonate solution (50cm^3). This solution was extracted with ethyl acetate (3 x 50cm^3) and the combined organic layers were washed with brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual yellow oil was purified over silica gel (80g) eluted with 0-30% methanol in ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (264) as a pale yellow oil (0.95g, 41%). TLC (methanol-ethyl acetate 1:3, $R_f = 0.4$); υ_{max} (film)/cm^{-1}: 2980m, 1720s, 1620w, 1500w, 1460w, 1400m, 1270m, 1195m, 1155w and 1090w; δ_H ($CDCl_3$)/ppm (90MHz) 8.13 (1H, dd, *J* 7.6 and 2.6, 6'-H), 7.63 (1H, td, *J* 7.6 and 2.6, 4'-H), 7.53 (1H, td, *J* 7.6 and 2.6, 5'-H), 7.25 (1H, dd, *J* 7.6 and 2.6, 3'-H), 4.35 (2H, t, *J* 6.3, 1-H_2), 3.23-2.96 (3H, m, 2"-H and 3"-H_2), 2.79 (2H, t, *J* 6.3, 2-H_2), 2.60 [4H, q, *J* 7.6, N(CH_2 CH_3)$_2$], 1.40 (3H, d, *J* 6.3, 5"-H_3) and 1.04 [6H, t, *J* 7.6, N(CH_2CH_3)$_2$]; δ_C ($CDCl_3$)/ppm (68MHz) 179.9 (C-1"), 176.0 (C-4"), 164.4 (OC=O), 133.4 (C-6'), 132.7 (C-2'), 131.6 (C-5'), 129.7 (C-4'), 129.3 (C-3'), 127.3 (C-1'), 63.5 (C-1), 50.9 (C-2), 47.6 [N(CH_2CH_3)$_2$], 37.0 (C-3"), 35.4 and 35.2 (C-2", both isomers), 16.5 and 16.3 (C-5", both isomers) and 11.9 [N(CH_2CH_3)$_2$]; $C_{18}H_{24}N_2O_4$ requires MW 332 and C, 65.0; H, 7.1; N, 8.4%. Found: m/z (+)FAB 333 (MH^+, 100%), (-)FAB 86 {[$CH_2N(CH_3)_2$]$^-$, 100%} and C, 64.9; H, 6.8; N, 8.3%. [See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.7 3-(*N,N*-Diethylamino)propyl 2-aminobenzoate (266) (Chemical Abstracts Registry Number [33709-03-4]).

To a stirred solution of isatoic anhydride (248) (4.08g, 25.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.30g, 2.5mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (40cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 3-(*N,N*-diethylamino)propan-1-ol (265) (3.25cm^3, 27.5mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (15h). The mixture was cooled to 25°C (45min) and then partitioned between ethyl acetate (70cm^3) and water (70cm^3). The aqueous layer was extracted with ethyl acetate (3 x 70cm^3) and the combined organic layers were then washed successively with water (3 x 70cm^3) and brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber oil was purified by distillation under reduced pressure (Bp 122°C/0.01mmHg) to give the 2-aminobenzoate (266) as a colourless oil (2.96g, 47%). TLC (methanol-ethyl acetate 1:3, $R_f = 0.1$); λ_{max} (EtOH)/nm: 248 (ε 11,000) and 337 (ε 6,700); υ_{max} (film)/cm^{-1}: 3490m, 2980s, 2820w, 1695s, 1625s, 1595m, 1460w, 1385m, 1250s and 1110w; δ_H ($CDCl_3$)/ppm (90MHz) 7.85 (1H, d, *J* 7.7, 6'-H), 7.23 (1H, t, *J* 7.7, 4'-H), 6.66-6.49 (2H, m, 3'- and 5'-H), 5.80 (2H, br s, NH_2), 4.30 (2H, t, *J* 6.4, 1-H_2), 2.57-2.41 [6H, m, 3-H_2 and N(CH_2 CH_3)$_2$], 1.87 (2H, quin, *J* 6.4, 2-H_2) and 1.01 [6H, t, *J* 6.4, N(CH_2CH_3)$_2$]; $C_{14}H_{22}N_2O_2$ requires MW 250 and C, 67.2; H, 8.9; N, 11.2%. Found: m/z (CI, isobutane) 251 (MH^+, 100%), 114 {[$CH_2CH_2CH_2N(CH_2CH_3)_2$]$^+$, 26%} and 86 {[$CH_2N(CH_2CH_3)_2$]$^+$, 94%} and C, 67.0; H, 9.1; N, 11.2%. [See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.8 3-(*N,N*-Diethylamino)propyl [2-(*RS*)-methylsuccinimido] benzoate (267)

A mixture of 2-aminobenzoate (266) (1.75g, 7.0mmol) and (*RS*)-methylsuccinic anhydride (257) (1.59, 14.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (4h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (50cm^3) and water (50cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (3 x 70cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange oil was purified over silica gel (80g) eluted with 0-30% methanol in ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (267) as a yellow oil (0.79g, 33%). TLC (methanol-ethyl acetate 1:3, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 2990w, 2850w, 1720s, 1610w, 1500w, 1460w, 1395m, 1300s, 1270m, 1200m, 1150w and 1110w; δ_H ($CDCl_3$)/ppm (90MHz) 8.12 (1H, dd, *J* 7.6 and 2.5, 6'-H), 7.62 (1H, td, *J* 7.6 and 2.5, 4'-H), 7.52 (1H, td, *J* 7.6 and 2.5, 5'-H), 7.26 (1H, dd, *J* 7.6 and 2.5, 3'-H), 4.26 (2H, t, *J* 6.3, 1-H_2), 3.20-2.88 (3H, m, 2"-H and 3"-H_2), 2.65-2.40 [6H, m, 3-H_2 and N(CH_2 CH_3)$_2$], 1.84 (2H, quin, *J* 6.3, 2-H_2), 1.44 (3H, d, *J* 7.5, 5"-H_3) and 1.00 [6H, t, *J* 7.5, N(CH_2CH_3)$_2$]; δ_C ($CDCl_3$)/ppm (68MHz) 179.8 (C-1"), 175.9 (C-4"), 164.3 (OC=O), 133.2 (C-5'), 132.6 (C-2'), 131.4 (C-6'), 129.7 (C-3'), 129.2 (C-4'), 127.2 (C-1'), 63.8 (C-1), 49.2 (C-3), 46.8 [N(CH_2CH_3)$_2$], 36.9 (C-3"), 35.3 and 35.1 (C-2", both isomers), 26.3 (C-2), 16.4 and 16.2 (C-5", both isomers) and 11.6 [N(CH_2CH_3)$_2$]; $C_{19}H_{26}N_2O_4$ requires MW 346 and C, 65.9; H, 7.6; N, 8.1%. Found: m/z (+)FAB 347 (MH^+, 100%), (-)FAB 346 (M^-, 5%) and 86 {[$CH_2N(CH_2CH_3)_2$]$^-$, 100%} and C, 65.7; H, 7.8; N, 8.0%.

[See Sections 4.2.2.2 and 4.2.3.2]

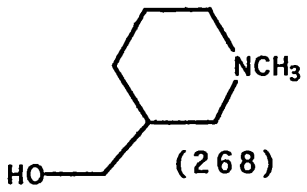
4.3.2.9 1-Methyl-3-(*RS*)-piperidinomethyl 2-aminobenzoate (269)

To a stirred solution of isatoic anhydride (248) (4.08g, 25.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.30g, 2.5mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (40cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 1-methyl-3-(*RS*)-piperidinomethanol (268) (3.55g, 27.5mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (7h). The mixture was cooled to 25°C (30min) and then partitioned between ethyl acetate (70cm^3) and water (70cm^3). The aqueous layer was extracted with ethyl acetate (3 x 70cm^3) and the combined organic layers were then washed successively with water (3 x 70cm^3) and brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber viscous oil was purified by distillation under reduced pressure (Bp 174-176°C/0.15mmHg) to give the 2-aminobenzoate (269) as a white crystalline solid (2.94g, 47%). Mp 60-61°C (methanol); TLC (methanol-dichloromethane 1:9, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 3480s, 2960w, 2790m, 1700s, 1620m, 1500w, 1380w, 1255m, 1110m and 340w; δ_H ($CDCl_3$)/ppm (270MHz) 7.85 (1H, dd, *J* 8.5 and 1.6, 6'-H), 7.25 (1H, td, *J* 7.6 and 1.6, 4'-H), 6.69-6.61 (2H, m, 3'- and 5'-H), 5.70 (2H, br s, NH_2), 4.16-4.12 (2H, m, $ArCO_2CH_2$ R), 2.93-2.87 (1H, m, 6_{eq}-H), 2.81 (1H, d, *J* 6.3, 2_{eq}-H), 2.30 (3H, s, NCH_3), 1.91-1.69 (6H, m, 2_{ax}-, 6_{ax}-, 5_{eq}-, 3-H and 4-H_2) and 1.22-1.20 (1H, m, 5_{ax}-H); $C_{14}H_{20}N_2O_2$ requires MW 248. Found: m/z (EI) 248 (M^+, 83%), 112 ($C_7H_{14}N^+$, 100%).

[See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.10 1-Methyl-3-(*RS*)-piperidinomethyl [2-(*RS*)-methylsuccinimido]benzoate (270)

A mixture of 2-aminobenzoate (269) (1.86g, 7.5mmol) and (*RS*)-methylsuccinic anhydride (257) (1.71g, 15.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (4h). The



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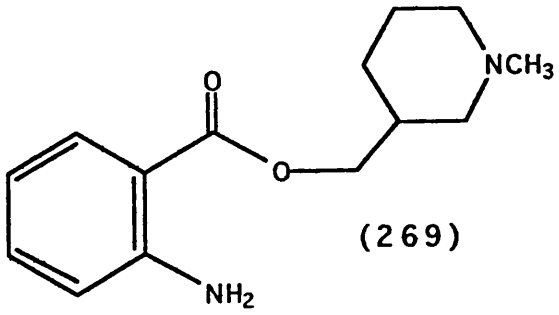

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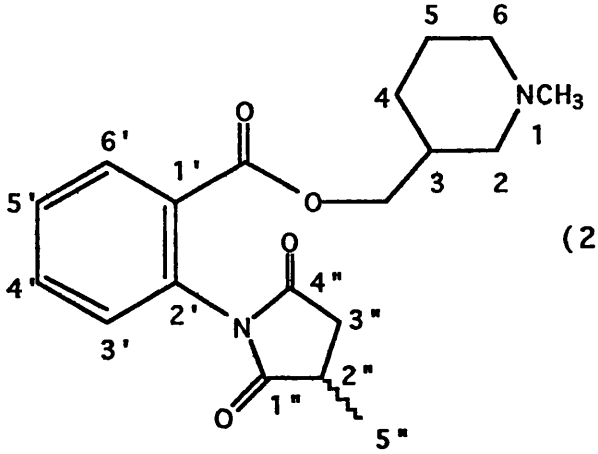

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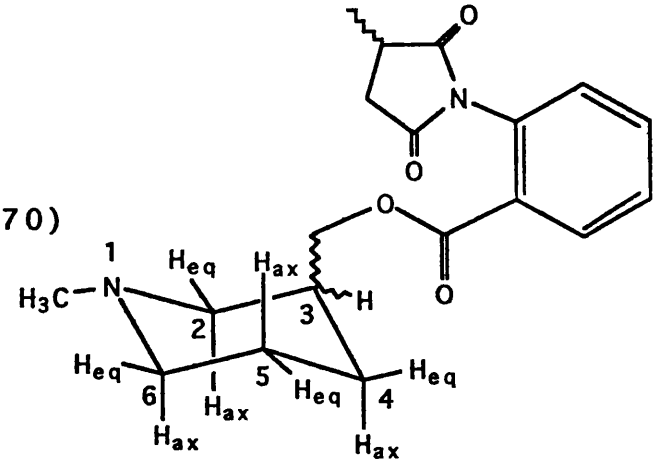

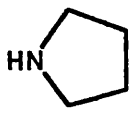

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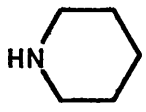

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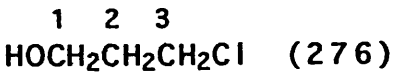

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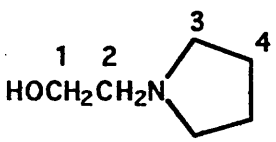

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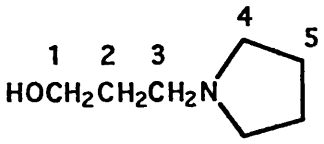

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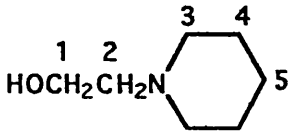

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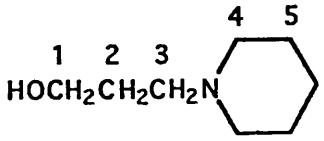


(284)

resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (50cm^3) and water (50cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (3 x 70cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange oil was purified over silica gel (100g) eluted with 10-40% methanol in dichloromethane to give the [2-(*RS*)-methylsuccinimido]benzoate (270) as a pale yellow oil (1.11g, 43%). TLC (methanol-dichloromethane 2:8, $R_f = 0.1$); υ_{max} (film)/cm^{-1}: 2965m, 2790m, 1800s, 1710s, 1620m, 1510m, 1270w and 1110m; δ_H ($CDCl_3$)/ppm (270MHz) 8.12 (1H, dd, *J* 7.6 and 2.5, 6'-H), 7.71 (1H, td, *J* 7.6 and 2.5, 4'-H), 7.51 (1H, td, *J* 7.6 and 2.5, 5'-H), 7.26 (1H, dd, *J* 7.6 and 2.5, 3'-H), 4.16-4.13 (2H, m, $ArCO_2CH_2$ R), 3.20-2.91 (3H, m, 6_{eq}-H and 3"-H_2), 2.55-2.52 (2H, m, 2_{eq}- and 2"-H), 2.33 (3H, s, NCH_3), 1.92-1.67 (6H, m, 2_{ax}-, 6_{ax}-, 5_{eq}-, 3-H and 4-H_2), 1.45 (3H, br d, *J* 7.5, 5"-H_3) and 1.15-1.11 (1H, m, 5_{ax}-H); δ_C ($CDCl_3$)/ppm (68MHz) 179.8 (C-1"), 176.0 (C-4"), 163.9 (OC=O), 133.4 (C-4'), 132.6 (C-2'), 131.5 (C-6'), 129.8 (C-3'), 129.2 (C-5'), 127.4 (C-1'), 70.1 ($ArCO_2CH_2R$), 60.5 (C-6), 58.7 (C-2 and C-5), 58.2 (C-4), 46.7 (C-3), 46.3 (NCH_3), 36.9 (C-3"), 35.4 and 35.2 (C-2", both isomers) and 16.5 and 16.2 (C-5", both isomers); $C_{19}H_{24}N_2O_4$ requires MW 344 and C, 66.3; H, 7.0; N, 8.1%. Found: m/z (+)FAB 345 (MH^+, 100%), (-)FAB 344 (M^-, 10%) and 112 ($C_7H_{14}N^-$, 100%) and C, 66.6; H, 6.9; N, 8.1%. [See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.11 2-*N*-Pyrrolidino-1-ethanol (273)
(Chemical Abstracts Registry Number [2955-88-6]).

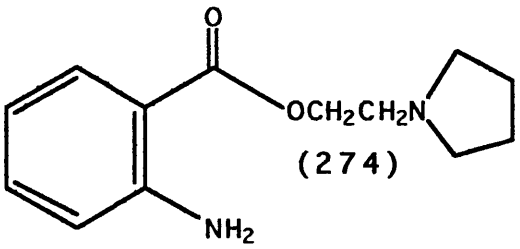
To a stirred solution of pyrrolidine (271) (7.10g, 100.0mmol, 2.0 equiv.) and a catalytic amount of sodium iodide (0.35g, 2.3mmol, 0.02 equiv.) in anhydrous absolute ethanol (100cm^3) at 25°C was added 2-chloro-1-ethanol (272) (4.00g, 50.0mmol) in one portion. The solution was then heated under reflux (oil-bath) and the stirring continued until completion of the reaction (24h). The mixture

was cooled to 25°C (1h) and then a solution of sodium (1.15g, 50.0mmol) in anhydrous absolute ethanol (200cm^3) was added with stirring over 30min. The mixture was then filtered in order to remove the precipitated sodium chloride and the residue was washed with diethyl ether (3 x 40cm^3). The filtrate was concentrated under reduced pressure to remove diethyl ether, ethanol and the excess of pyrrolidine. The residue was diluted with diethyl ether (15cm^3) and filtered. The filtrate was dried ($MgSO_4$) and concentrated under reduced pressure. The residual yellow mobile oil was purified by distillation under reduced pressure {Bp 107-109°C/~20mmHg [lit. Bp 78-79°C/18mmHg (Leonard and Musker, 1960)]} to give the tertiary amino primary alcohol (273) as a colourless oil (4.49g, 78%). TLC (methanol-dichloromethane 2:8, R_f = 0.2); υ_{max} (film)/cm^{-1}: 3403m, 2942w, 2801m, 2581w, 1460w, 1379w and 719w; δ_H ($CDCl_3$)/ppm (270MHz) 4.70 (1H, br s, OH), 3.75 (2H, t, *J* 6.3, 1-H_2), 2.78 (2H, t, *J* 6.3, 2-H_2), 2.60-2.49 (4H, m, 2 x 3-H_2) and 1.82-1.73 (4H, m, 2 x 4-H_2); $C_6H_{13}NO$ requires MW 115. Found: m/z (EI) 115 (M^+, 100%).

[See Section 4.2.3.3]

4.3.2.12 2-*N*-Pyrrolidinoethyl 2-aminobenzoate (274)
(Chemical Abstracts Registry Number [88562-50-9]).

To a stirred solution of isatoic anhydride (248) (4.89g, 30.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.36g, 3.0mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (50cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 2-*N*-pyrrolidino-1-ethanol (273) (3.80g, 33.0mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (3h). The mixture was cooled to 25°C (20min) and then partitioned between ethyl acetate (75cm^3) and water (75cm^3). The aqueous layer was extracted with ethyl acetate (3 x 75cm^3) and the combined organic layers were then washed successively with water (3 x 50cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber oil was purified by distillation



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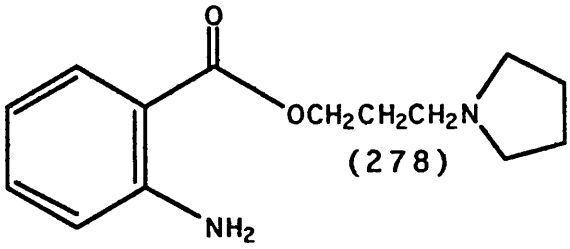

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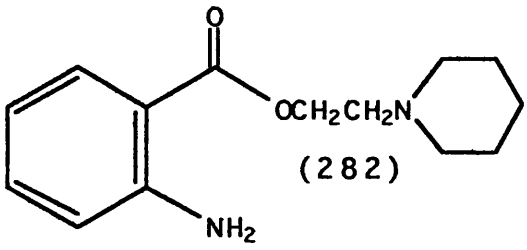

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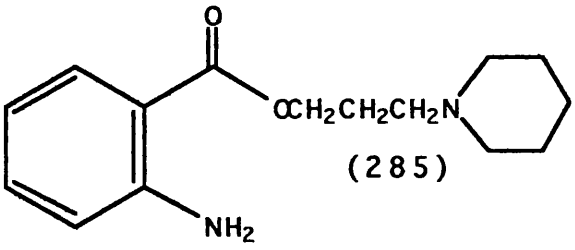

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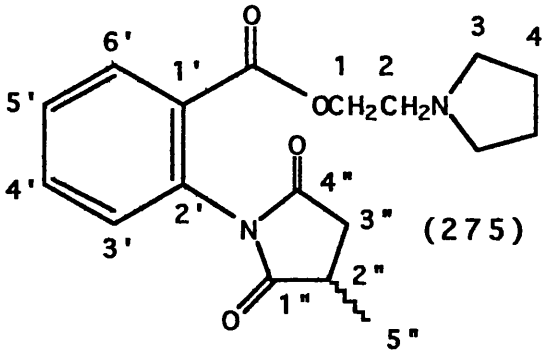

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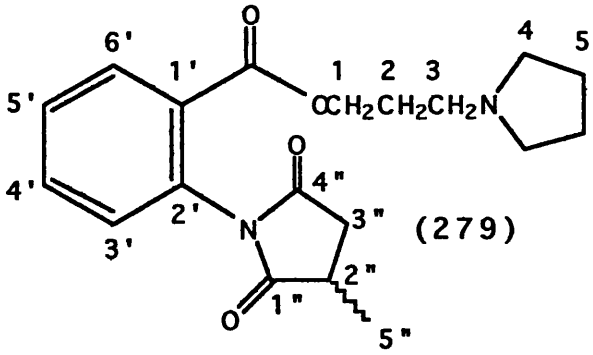

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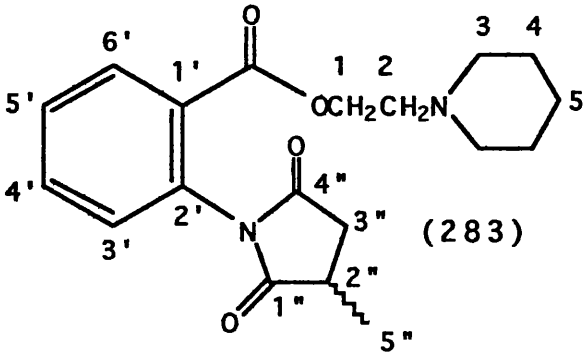

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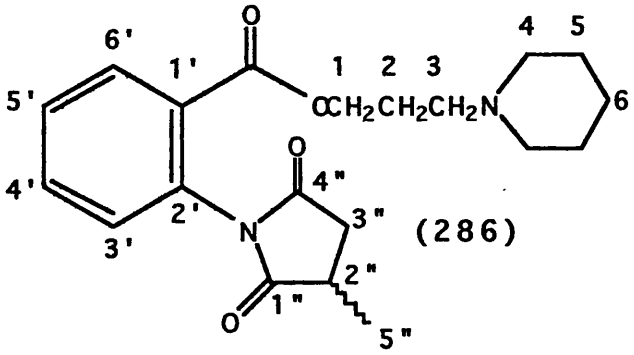


(286)

under reduced pressure (Bp 136-138°C/0.01mmHg) to give the 2-aminobenzoate (274) as a colourless oil (4.35g, 62%). TLC (methanol-dichloromethane 1:9, $R_f = 0.5$); υ_{max} (film)/cm^{-1}: 3491s, 3389s, 3155m, 2951s, 2899m, 2500m, 1780s, 1720s, 1452m, 1250s and 720w; δ_H ($CDCl_3$)/ppm (270MHz) 7.84 (1H, dd, *J* 7.8 and 2.3, 6'-H), 7.21 (1H, td, *J* 7.8 and 1.9, 4'-H), 6.69-6.59 (2H, m, 3'- and 5'-H), 5.72 (2H, br s, NH_2), 4.32 (2H, t, *J* 6.2, 1-H_2), 2.64 (2H, t, *J* 6.2, 2-H_2), 2.55-2.50 (4H, m, 2 x 3-H_2) and 1.75 (4H, t, *J* 6.5, 2 x 4-H_2); $C_{13}H_{18}N_2O_2$ requires MW 234. Found: m/z (EI) 234 (M^+, 100%) and 84 {$[CH_2N{=}(CH_2)_4]^+$, 60%}. [See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.13 2-*N*-Pyrrolidinoethyl [2-(*RS*)-methylsuccinimido]benzoate (275)

A mixture of 2-aminobenzoate (274) (2.80g, 12.0mmol) and (*RS*)-methylsuccinic anhydride (257) (2.82g, 24.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (3h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (100cm^3) and saturated aqueous sodium hydrogen carbonate solution (100cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (3 x 75cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange gum was purified over silica gel (140g) eluted with 0-30% methanol in ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (275) as a yellow oil (2.18g, 55%). TLC (methanol-ethyl acetate 2:8, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 2954s, 2800m, 1782s, 1725s, 1709s, 1620s, 1500m, 1460w, 1390s, 1260s and 721w; δ_H ($CDCl_3$)/ppm (270MHz) 8.13 (1H, dd, *J* 7.6 and 2.5, 6'-H), 7.63 (1H, td, *J* 7.6 and 2.5, 4'-H), 7.51 (1H, td, *J* 7.6 and 2.5, 5'-H), 7.25 (1H, dd, *J* 7.6 and 2.5, 3'-H), 4.35 (2H, t, *J* 5.7, 1-H_2), 3.36-2.92 (3H, m, 2"-H and 3"-H_2), 2.68 (2H, t, *J* 5.7, 2-H_2), 2.55 (4H, t, *J* 6.1, 2 x 3-H_2), 1.81 (4H, t, *J* 6.1, 2 x 4-H_2) and 1.52 (3H, d, *J* 6.3, 5"-H_3); δ_C ($CDCl_3$)/ppm (68MHz) 179.7 (C-1"), 175.8 (C-4"), 164.2 (OC=O), 133.4

(C-4'), 132.8 (C-2'), 131.5 (C-6'), 129.9 (C-3'), 129.4 (C-5'), 127.5 (C-1'), 63.0 (C-1), 55.7 (C-3), 53.6 (C-2), 52.7 (C-4), 37.1 (C-3"), 35.3 and 35.1 (C-2", both isomers) and 16.5 and 16.3 (C-5", both isomers); $C_{18}H_{22}N_2O_4$ requires MW 330 and C, 65.4; H, 6.7; N, 8.5%. Found: m/z (+)FAB 331 (MH^+, 100%), (-)FAB 84 {$[CH_2N=(CH_2)_4]^-$, 100%} and C, 65.7; H, 6.8; N, 8.4%.

[See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.14 3-*N*-Pyrrolidino-1-propanol (277)
(Chemical Abstracts Registry Number [19748-66-4]).

To a stirred solution of pyrrolidine (271) (7.10g, 100.0mmol, 2.0 equiv.) and a catalytic amount of sodium iodide (0.35g, 2.3mmol, 0.02 equiv.) in anhydrous absolute ethanol (100cm^3) at 25°C was added 3-chloro-1-propanol (276) (4.73g, 50.0mmol) in one portion. The solution was then heated under reflux (oil-bath) and the stirring continued until completion of the reaction (24h). The mixture was cooled to 25°C (1h) and then a solution of sodium (1.15g, 50.0mmol) in anhydrous absolute ethanol (200cm^3) was added with stirring over 30min. The mixture was then filtered in order to remove the precipitated sodium chloride and the residue was washed with diethyl ether (3 x 40cm^3). The filtrate was concentrated under reduced pressure to remove diethyl ether, ethanol and the excess of pyrrolidine. The residue was diluted with diethyl ether (15cm^3) and filtered. The filtrate was dried ($MgSO_4$) and concentrated under reduced pressure. The residual yellow mobile oil was purified by distillation under reduced pressure {Bp 124-126°C/~20mmHg [lit. Bp 100-101°C/15mmHg (Leonard and Musker, 1960)]} to give the tertiary amino primary alcohol (277) as a colourless oil (4.06g, 63%). TLC (methanol-dichloromethane 2:8, $R_f = 0.1$); υ_{max} (film)/cm^{-1}: 3400m, 2940w, 2800m, 1630m, 1595w, 1460w, 1380m, 1250s and 1110m; δ_H ($CDCl_3$)/ppm (270MHz) 4.90 (1H, br s, OH), 3.78 (2H, t, *J* 6.3, 1-H_2), 2.73 (2H, t, *J* 6.3, 3-H_2), 2.59-2.52 (4H, m, 2 x 4-H_2) and 1.83-1.71 (6H, m, 2-H_2 and 2 x 5-H_2); $C_7H_{15}NO$ requires MW 129. Found: m/z (EI) 129 (M^+, 100%). [See Section 4.2.3.3]

4.3.2.15 3-*N*-Pyrrolidinopropyl 2-aminobenzoate (278)

To a stirred solution of isatoic anhydride (248) (3.26g, 20.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.24g, 2.0mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (32cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 3-*N*-pyrrolidino-1-propanol (277) (2.84g, 22.0mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (3h). The mixture was cooled to 25°C (20min) and then partitioned between ethyl acetate (50cm^3) and water (50cm^3). The aqueous layer was extracted with ethyl acetate (3 x 50cm^3) and the combined organic layers were then washed successively with water (3 x 50cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber oil was purified by distillation under reduced pressure (Bp 140-142°C/0.01mmHg) to give the 2-aminobenzoate (278) as a colourless oil (4.54g, 92%). TLC (methanol-dichloromethane 1:9, $R_f = 0.4$); υ_{max} (film)/cm^{-1}: 3490s, 3390s, 3155w, 2950s, 2800m, 1700s, 1620s, 1490w, 1450w, 1370m, 1250s and 1110m; δ_H ($CDCl_3$)/ppm (270MHz) 7.85 (1H, dd, *J* 8.1 and 1.3, 6'-H), 7.24 (1H, td, *J* 7.7 and 1.7, 4'-H), 6.68-6.60 (2H, m, 3'- and 5'-H), 5.75 (2H, br s, NH_2), 4.32 (2H, t, *J* 6.4, 1-H_2), 2.61 (2H, t, *J* 7.8, 3-H_2), 2.52 (4H, t, *J* 6.3, 2 x 4-H_2), 1.98 (2H, t, *J* 7.1, 2-H_2) and 1.78 (4H, t, *J* 6.3, 2 x 5-H_2); $C_{14}H_{20}N_2O_2$ requires MW 248. Found: m/z (EI) 248 (M^+, 100%) and 84 {$[CH_2N{=}(CH_2)_4]^+$, 90%}.

[See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.16 3-*N*-Pyrrolidinopropyl [2-(*RS*)-methylsuccinimido]benzoate (279)

A mixture of 2-aminobenzoate (278) (1.50g, 6.0mmol) and (*RS*)-methylsuccinic anhydride (257) (1.41g, 12.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (3h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned

between ethyl acetate (50cm^3) and saturated aqueous sodium hydrogen carbonate solution (50cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (3 x 50cm^3) and brine (40cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange gum was purified over silica gel (70g) eluted with 0-20% methanol in ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (279) as a yellow oil (1.12g, 56%). TLC (methanol-ethyl acetate 3:7, R_f = 0.8); υ_{max} (film)/cm^{-1}: 2990m, 2800m, 1710s, 1620s, 1500m, 1460m, 1400s, 1370m, 1260s and 1110w; δ_H ($CDCl_3$)/ppm (270MHz) 8.12 (1H, dd, *J* 7.6 and 2.5, 6'-H), 7.65 (1H, td, *J* 7.6 and 2.5, 4'-H), 7.55 (1H, td, *J* 7.6 and 2.5, 5'-H), 7.24 (1H, dd, *J* 7.6 and 2.5, 3'-H), 4.30 (2H, t, *J* 6.4, 1-H_2), 3.36-2.96 (3H, m, 2"-H and 3"-H_2), 2.65 (2H, t, *J* 5.7, 3-H_2), 2.55 (4H, t, *J* 6.1, 2 x 4-H_2), 2.02 (2H, t, *J* 6.4, 2-H_2), 1.80 (4H, t, *J* 6.1, 2 x 5-H_2) and 1.53 (3H, d, *J* 6.3, 5"-H_3); δ_C ($CDCl_3$)/ppm (68MHz) 179.8 (C-1"), 176.0 (C-4"), 163.8 (OC=O), 133.3 (C-4'), 132.6 (C-2'), 131.7 (C-6'), 130.0 (C-3'), 129.1 (C-5'), 127.4 (C-1'), 63.5 (C-1), 56.4 (C-4), 54.6 (C-5), 53.4 (C-3), 37.1 (C-3"), 35.4 and 35.1 (C-2", both isomers), 26.5 (C-2) and 16.4 and 16.1 (C-5", both isomers); $C_{19}H_{24}N_2O_4$ requires MW 344 and C, 66.3; H, 7.0; N, 8.1%. Found: m/z (+)FAB 345 (MH^+, 100%), (-)FAB 84 {$[CH_2N{=}(CH_2)_4]^-$, 100%} and C, 66.4; H, 7.1; N, 8.0%.

[See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.17 2-*N*-Piperidino-1-ethanol (281)

(Chemical Abstracts Registry Number [3040-44-6]).

To a stirred solution of piperidine (280) (8.50g, 100.0mmol, 2.0 equiv.) and a catalytic amount of sodium iodide (0.35g, 2.3mmol, 0.02 equiv.) in anhydrous absolute ethanol (100cm^3) at 25°C was added 2-chloro-1-ethanol (272) (4.03g, 50.0mmol) in one portion. The solution was then heated under reflux (oil-bath) and the stirring continued until completion of the reaction (24h). The mixture was cooled to 25°C (1h) and then a solution of sodium (1.15g, 50.0mmol) in anhydrous absolute ethanol (200cm^3) was added with stirring over 30min. The

mixture was then filtered in order to remove the precipitated sodium chloride and the residue was washed with diethyl ether (3 x 40cm^3). The filtrate was concentrated under reduced pressure to remove diethyl ether, ethanol and the excess of piperidine. The residue was diluted with diethyl ether (15cm^3) and filtered. The filtrate was dried ($MgSO_4$) and concentrated under reduced pressure. The residual yellow mobile oil was purified by distillation under reduced pressure {Bp 115-118°C/~25mmHg [lit. Bp 95-96°C/20mmHg (Leonard and Musker, 1960)]} to give the tertiary amino primary alcohol (281) as a colourless oil (4.20g, 65%). TLC (methanol-dichloromethane 1:9, $R_f = 0.1$); υ_{max} (film)/cm^{-1}: 3398m, 2938w, 2800s, 1461w, 1381w, 1110m and 722w; δ_H ($CDCl_3$)/ppm (270MHz) 4.62 (1H, br s, OH), 3.76 (2H, t, *J* 6.3, 1-H_2), 2.80-2.75 (6H, m, 2 x 3-H_2 and 2-H_2), 1.56-1.51 (6H, m, 2 x 4-H_2 and 5-H_2); $C_7H_{15}NO$ requires MW 129. Found: m/z (EI) 129 (M^+, 100%).

[See Section 4.2.3.3]

4.3.2.18 2-*N*-Piperidinoethyl 2-aminobenzoate (282)
(Chemical Abstracts Registry Number [88562-51-0]).

To a stirred solution of isatoic anhydride (248) (3.26g, 20.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.24g, 2.0mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (32cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 2-*N*-piperidino-1-ethanol (281) (2.84g, 22.0mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (3h). The mixture was cooled to 25°C (20min) and then partitioned between ethyl acetate (50cm^3) and water (50cm^3). The aqueous layer was extracted with ethyl acetate (3 x 50cm^3) and the combined organic layers were then washed successively with water (3 x 50cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber oil was purified by distillation under reduced pressure (Bp 139-141°C/0.04mmHg) to give the 2-aminobenzoate (282) as a colourless oil (3.47g, 70%). TLC (dichloromethane, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 3492s, 3390s, 3153w, 2950s, 2803m, 1621s, 1450w,

1372m, 1110m and 719w; δ_H ($CDCl_3$)/ppm (270MHz) 7.75 (1H, dd, *J* 8.1 and 2.3, 6'-H), 7.25 (1H, td, *J* 8.1 and 2.3, 4'-H), 6.78-6.58 (2H, m, 3'- and 5'-H), 5.90 (2H, br s, NH_2), 4.35 (2H, t, *J* 7.0, 1-H_2), 2.70 (4H, t, *J* 6.3, 2 x 3-H_2), 2.63 (2H, t, *J* 7.0, 2-H_2) and 1.52-1.48 (6H, m, 5-H_2 and 2 x 4-H_2); $C_{14}H_{20}N_2O_2$ requires MW 248. Found: m/z (EI) 248 (M^+, 100%) and 96 {$[CH_2N{=}(CH_2)_5]^+$, 78%}. [See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.19 2-*N*-Piperidinoethyl [2-(*RS*)-methylsuccinimido]benzoate (283)

A mixture of 2-aminobenzoate (282) (2.48g, 10.0mmol) and (*RS*)-methylsuccinic anhydride (257) (2.35g, 20.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (3h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (75cm^3) and saturated aqueous sodium hydrogen carbonate solution (75cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (3 x 75cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange gum was purified over silica gel (115g) eluted with ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (283) as a yellow oil (2.00g, 58%). TLC (ethyl acetate, R_f = 0.3); υ_{max} (film)/cm^{-1}: 2955m, 2800s, 1779s, 1725s, 1709s, 1620s, 1459w, 1375m, 1260s and 722w; δ_H ($CDCl_3$)/ppm (270MHz) 8.13 (1H, dd, *J* 7.6 and 2.6, 6'-H), 7.65 (1H, td, *J* 7.6 and 2.6, 4'-H), 7.53 (1H, td, *J* 7.6 and 2.6, 5'-H), 7.24 (1H, dd, *J* 7.6 and 2.6, 3'-H), 4.35 (2H, t, *J* 6.4, 1-H_2), 3.23-2.96 (3H, m, 2"-H and 3"-H_2), 2.81-2.76 (4H, m, 2 x 3-H_2), 2.68 (2H, t, *J* 6.3, 2-H_2), 1.56-1.51 (6H, m, 5-H_2 and 2 x 4-H_2) and 1.43 (3H, d, *J* 6.3, 5"-H_3); δ_C ($CDCl_3$)/ppm (68MHz) 180.0 (C-1"), 176.2 (C-4"), 164.4 (OC=O), 63.8 (C-1), 55.4 (C-2), 56.3 (C-3), 53.8 (C-4 and C-5), 133.3 (C-4'), 132.9 (C-2'), 131.4 (C-6'), 129.7 (C-3'), 129.5 (C-5'), 127.3 (C-1'), 36.9 (C-3"), 35.4 and 35.2 (C-2", both isomers) and 16.4 and 16.2 (C-5", both isomers); $C_{19}H_{24}N_2O_4$ requires MW 344 and C, 66.3; H, 7.0; N, 8.1%. Found: m/z (+)FAB 345 (MH^+, 100%), (-)FAB 96 {$[CH_2N{=}(CH_2)_5]^-$, 100%} and C, 66.1; H, 7.0; N, 8.1%. [See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.20 3-*N*-Piperidino-1-propanol (284)
(Chemical Abstracts Registry Number [104-58-5]).

To a stirred solution of piperidine (280) (8.50g, 100.0mmol, 2.0 equiv.) and a catalytic amount of sodium iodide (0.35g, 2.3mmol, 0.02 equiv.) in anhydrous absolute ethanol ($100cm^3$) at 25°C was added 3-chloro-1-propanol (276) (4.73g, 50.0mmol) in one portion. The solution was then heated under reflux (oil-bath) and the stirring continued until completion of the reaction (24h). The mixture was cooled to 25°C (1h) and then a solution of sodium (1.15g, 50.0mmol) in anhydrous absolute ethanol ($200cm^3$) was added with stirring over 30min. The mixture was then filtered in order to remove the precipitated sodium chloride and the residue was washed with diethyl ether ($3 \times 40cm^3$). The filtrate was concentrated under reduced pressure to remove diethyl ether, ethanol and the excess of piperidine. The residue was diluted with diethyl ether ($15cm^3$) and filtered. The filtrate was dried ($MgSO_4$) and concentrated under reduced pressure. The residual yellow mobile oil was purified by distillation under reduced pressure {Bp 133-135°C/~20mmHg [lit. Bp 114°C/19mmHg (Leonard and Musker)]} to give the tertiary amino primary alcohol (284) as a colourless oil (5.72g, 80%). TLC (methanol-dichloromethane 2:8, $R_f = 0.1$); υ_{max} (film)/cm^{-1}: 3395m, 2940s, 2800s, 2581w, 1463w, 1380m and 718w; δ_H ($CDCl_3$)/ppm (270MHz) 4.90 (1H, br s, OH), 3.79 (2H, t, *J* 6.3, 1-H_2), 2.81-2.71 (6H, m, 3-H_2 and 2 x 4-H_2), 1.83-1.71 (2H, m, 2-H_2) and 2.59-2.52 (6H, m, 6-H_2 and 2 x 5-H_2); $C_8H_{17}NO$ requires MW 143. Found: m/z (EI) 143 (M^+, 100%).

[See Section 4.2.3.3]

4.3.2.21 3-*N*-Piperidinopropyl 2-aminobenzoate (285)

To a stirred solution of isatoic anhydride (248) (4.08g, 25.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.3g, 2.5mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide ($40cm^3$) at 25°C, under an atmosphere of anhydrous nitrogen, was added 3-*N*-piperidino-1-propanol (284) (3.94g,

27.5mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (3h). The mixture was cooled to 25°C (20min) and then partitioned between ethyl acetate ($60cm^3$) and water ($60cm^3$). The aqueous layer was extracted with ethyl acetate (3 x $50cm^3$) and the combined organic layers were then washed successively with water (3 x $50cm^3$) and brine ($40cm^3$), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber oil was purified by distillation under reduced pressure (Bp 154-156°C/0.01mmHg) to give the 2-aminobenzoate (285) as a colourless oil (4.32g, 66%). TLC (methanol-dichloromethane 2:8, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 3490s, 3395m, 3156w, 2950s, 2801m, 1720s, 1620m, 1453w, 1252m and 710m; δ_H ($CDCl_3$)/ppm (270MHz) 7.85 (1H, dd, *J* 7.7 and 1.3, 6'-H), 7.23 (1H, td, *J* 7.7 and 1.3, 4'-H), 6.66-6.49 (2H, m, 3'- and 5'-H), 5.75 (2H, br s, NH_2), 4.28 (2H, t, *J* 6.4, 1-H_2), 2.70 (4H, t, *J* 7.8, 4-H_2), 2.45 (2H, t, *J* 6.3, 3-H_2), 1.85 (2H, quin, *J* 6.3, 2-H_2) and 1.52-1.48 (6H, m, 6-H_2 and 2 x 5-H_2); $C_{15}H_{22}N_2O_2$ requires MW 262. Found: m/z (EI) 262 (M^+, 100%) and 96 {$[CH_2N{=}(CH_2)_5]^+$, 95%}.

[See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.22 3-*N*-Piperidinopropyl [2-(*RS*)-methylsuccinimido]benzoate (286)

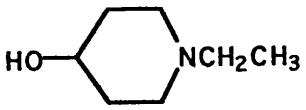
A mixture of 2-aminobenzoate (285) (3.14g, 12.0mmol) and (*RS*)-methylsuccinic anhydride (257) (2.82g, 24.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (3h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate ($100cm^3$) and saturated aqueous sodium hydrogen carbonate solution ($100cm^3$). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (3 x $100cm^3$) and brine ($80cm^3$), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange gum was purified over silica gel (140g) eluted with 0-20% methanol in dichloromethane to give the [2-(*RS*)-

methylsuccinimido]benzoate (286) as a yellow oil (3.06g, 74%). TLC (methanol-dichloromethane 2:8, $R_f = 0.2$); υ_{max} (film)/cm^{-1}: 2960m, 2802m, 1785s, 1725s, 1708s, 1504w, 1459m, 1402m, 1110w and 720w; δ_H ($CDCl_3$)/ppm (270MHz) 8.12 (1H, dd, *J* 7.6 and 2.5, 6'-H), 7.62 (1H, td, *J* 7.6 and 2.5, 4'-H), 7.52 (1H, td, *J* 7.6 and 2.5, 5'-H), 7.24 (1H, dd, *J* 7.6 and 2.5, 3'-H), 4.28 (2H, t, *J* 6.4, 1-H_2), 3.24-2.92 (3H, m, 2"-H and 3"-H_2), 2.77-2.73 (4H, m, 2 x 4-H_2), 2.50 (2H, t, *J* 5.7, 3-H_2), 1.97-1.93 (2H, m, 2-H_2), 1.53-1.49 (6H, m, 6-H_2 and 2 x 5-H_2) and 1.43 (3H, d, *J* 6.3, 5"-H_3); δ_C ($CDCl_3$)/ppm (68MHz) 179.9 (C-1"), 176.2 (C-4"), 163.9 (OC=O), 133.4 (C-4'), 133.1 (C-3'), 132.6 (C-2'), 131.4 (C-6'), 129.3 (C-5'), 127.6 (C-1'), 63.2 (C-1), 57.9 (C-4), 55.8 (C-5), 55.0 (C-6), 50.3 (C-3), 37.2 (C-3"), 35.4 and 35.2 (C-2", both isomers), 26.9 (C-2) and 16.4 and 16.2 (C-5", both isomers); $C_{20}H_{26}N_2O_4$ requires MW 358 and C, 67.0; H, 7.3; N, 7.8%. Found: m/z (+)FAB 359 (MH^+, 100%), (-)FAB 96 {$[CH_2N=(CH_2)_5]^-$, 100%} and C, 66.0; H, 7.2; N, 7.8%.

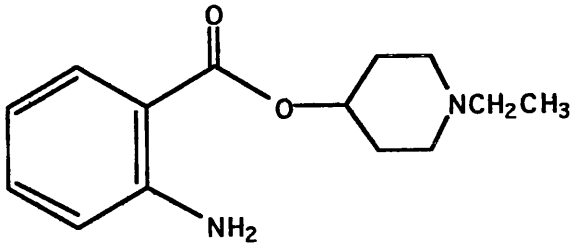
[See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.23 1-Ethyl-4-piperidino 2-aminobenzoate (288)

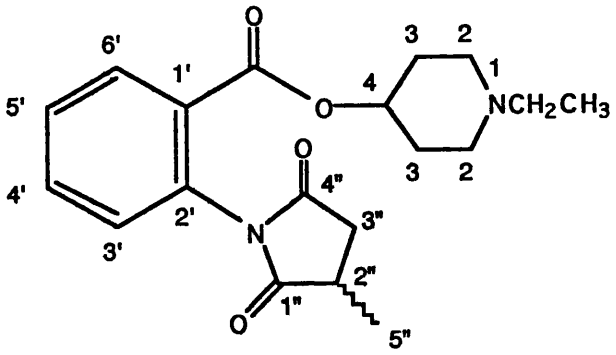
To a stirred solution of isatoic anhydride (248) (4.08g, 25.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.30g, 2.5mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (40cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added 1-ethyl-4-piperidinol (287) (3.55g, 27.5mmol, 1.1 equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (7h). The mixture was cooled to 25°C (30min) and then partitioned between ethyl acetate (70cm^3) and water (70cm^3). The aqueous layer was extracted with ethyl acetate (3 x 70cm^3) and the combined organic layers were then washed successively with water (3 x 70cm^3) and brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual amber viscous oil was purified by distillation under reduced pressure (169-171°C/0.10mmHg) to give the 2-aminobenzoate (288) as a white crystalline solid (2.04g, 33%). Mp 252-254°C; TLC (methanol-


HO
NCH2CH3

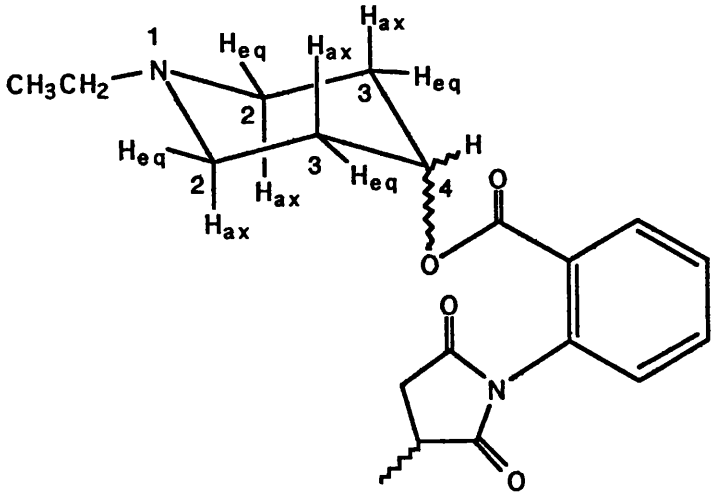
(287)


O
O
NCH2CH3
NH2

(288)


O
6'
1'
3
2
1
4
5'
O
NCH2CH3
3
2
O
4"
4'
2'
N
3"
3'
2"
O
1"
5"

(289)


Hax
1
Heq
Hax
CH3CH2
N
Heq
3
2
Heq
H
3
2
Hax
Heq
4
O
Hax
O
O
N
O

dichloromethane 2:8, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 3480s, 3026w, 2960m, 2894s, 2790m, 2453m, 1724s, 1610m, 1579m, 1381s, 1452m, 754s and 723w; δ_H ($CDCl_3$)/ppm (270MHz) 7.86 (1H, dd, *J* 8.5 and 1.6, 6'-H), 7.25 (1H, td, *J* 7.6 and 1.6, 4'-H), 6.70-6.62 (2H, m, 3'- and 5'-H), 5.70 (2H, br s, NH_2), 4.88-4.63 (1H, m, 4-H), 2.60-2.48 (8H, m, 2 x 3_{ax}-H, 2 x 2-H_2 and NCH_2 CH_3), 2.27-2.24 (2H, m, 2 x 3_{eq}-H) and 1.10 (3H, t, *J* 7.2, NCH_2CH_3); $C_{14}H_{20}N_2O_2$ requires MW 248. Found: m/z (EI) 248 (M^+, 66%), 112 ($C_7H_{14}N^+$, 100%).

[See Sections 4.2.2.1 and 4.2.3.1]

4.3.2.24 1-Ethyl-4-piperidino [2-(*RS*)-methylsuccinimido]benzoate (289)

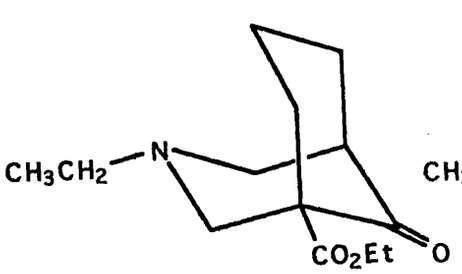
A mixture of 2-aminobenzoate (288) (1.86g, 7.5mmol) and (*RS*)-methylsuccinic anhydride (257) (1.71g, 15.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (4h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (50cm^3) and water (50cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (3 x 70cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange oil was purified over silica gel (100g) eluted with 0-30% methanol in ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (289) as a pale yellow oil (0.88g, 34%). TLC (methanol-ethyl acetate 3:7, $R_f = 0.2$); υ_{max} (film)/cm^{-1}: 3028w, 2890m, 2895s, 2790m, 1770s, 1723s, 1700s, 1612m, 1510m, 1382s, 1453m, 755s and 720w; δ_H ($CDCl_3$)/ppm (270MHz) 8.12 (1H, dd, *J* 7.6 and 2.5, 6'-H), 7.71 (1H, td, *J* 7.6 and 2.5, 4'-H), 7.51 (1H, td, *J* 7.6 and 2.5, 5'-H), 7.26 (1H, dd, *J* 7.6 and 2.5, 3'-H), 4.90-4.78 (1H, m, 4-H), 3.11-3.02 (2H, m, 3"-H_2), 2.61-2.47 (9H, m, 2"-H, 2 x 3_{ax}-H, 2 x 2-H_2 and NCH_2 CH_3), 2.28-2.23 (2H, m, 2 x 3_{eq}-H), 1.42 (3H, br d, *J* 7.5, 5"-H_3) and 1.11 (3H, t, *J* 7.3, NCH_2CH_3); δ_C ($CDCl_3$)/ppm (68MHz) 179.8 (C-1"), 176.1 (C-4"), 164.0 (OC=O), 133.2 (C-4'), 132.7 (C-2'), 131.5 (C-6'), 129.8 (C-3'), 129.2 (C-5'), 127.2 (C-1'), 60.1 (C-4), 55.9 (C-2), 53.5 (C-3), 52.1

(NCH_2CH_3), 36.9 (C-3"), 35.3 and 35.1 (C-2", both isomers), 16.5 and 16.3 (C-5", both isomers) and 12.7 (NCH_2CH_3); $C_{19}H_{24}N_2O_4$ requires MW 344 and C, 66.3; H, 7.0; N, 8.1%. Found: m/z (+)FAB 345 (MH^+, 100%), (-)FAB 344 (M^-, 58%) and 112 ($C_7H_{14}N^-$, 100%) and C, 66.2; H, 6.9; N, 8.1%.

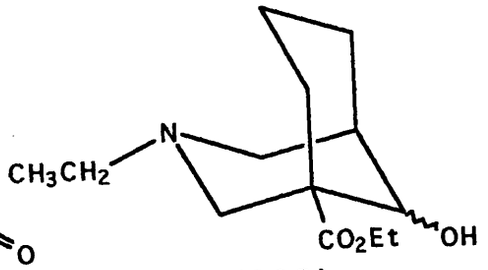
[See Sections 4.2.2.2 and 4.2.3.2]

4.3.2.25 Ethyl 3-ethyl-3-aza-bicyclo[3.3.1]nonan-9-one-1-carboxylate (292)

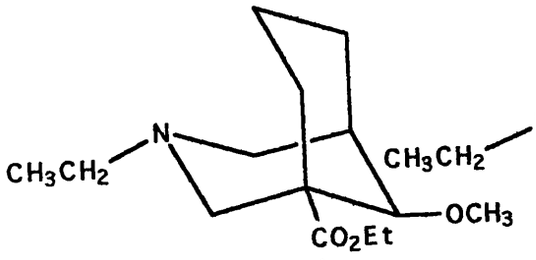
To a stirred solution of 70% aqueous ethylamine (290) (7.5cm^3, 90mmol) in ethanol (20cm^3) at 25°C was added dropwise (15min) 37% aqueous formaldehyde (15.0cm^3, 185mmol, 2.0 equiv.). The resulting solution was added in one portion to a stirred solution of ethyl 2-cyclohexanone-1-carboxylate (291) (15.3g, 90mmol) in ethanol (50cm^3) at 25°C. Glacial acetic acid (1.0cm^3) was added to the solution which was then heated under reflux (oil-bath), under an atmosphere of nitrogen, and the stirring continued until completion of the reaction (2h). The reaction mixture was cooled to 25°C (16h), concentrated under reduced pressure and then diluted with ethyl acetate (160cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (100cm^3) and aqueous hydrochloric acid solution (2M, 2 x 100cm^3). The combined acidic layers were extracted with ethyl acetate (70cm^3) and then basified to pH 9 (solid sodium hydrogen carbonate then 2M aqueous sodium hydroxide solution). The mixture was then extracted with ethyl acetate (2 x 100cm^3) and the combined organic layers were washed with brine (2 x 50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange oil was purified over silica gel (125g) eluted with diethyl ether-hexane (1:1) to give a golden oil which was further purified by distillation under reduced pressure (Bp 110-112°C/0.35mmHg) to give the ketoester (292) as a colourless oil (10.1g, 47%). TLC (diethyl ether-hexane 1:1, $R_f = 0.4$); υ_{max} (film)/cm^{-1}: 2931m, 1737s, 1735s, 1455w, 1367w, 1256s and 1057m; δ_H ($CDCl_3$)/ppm (270MHz) 4.21 (2H, q, *J* 7.2, CO_2CH_2 CH_3), 3.22 (1H, dd, *J* 10.9 and 1.0, 2_{eq}-H), 3.16 (1H, dt, *J* 10.9 and 0.9, 2_{ax}-H), 2.93 (1H,



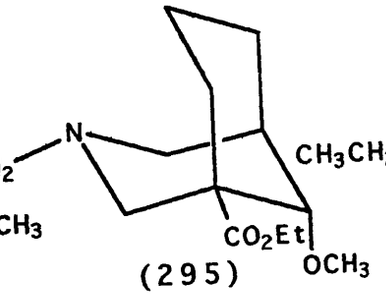
(292)



(293)



(294)



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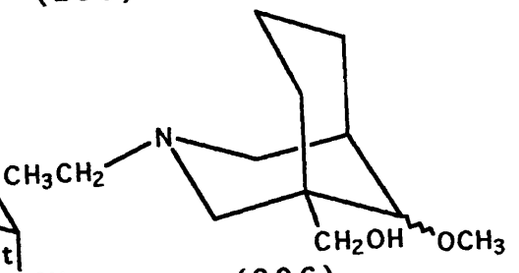

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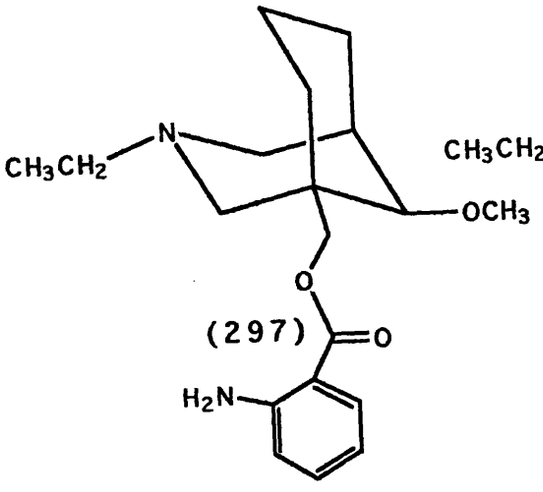

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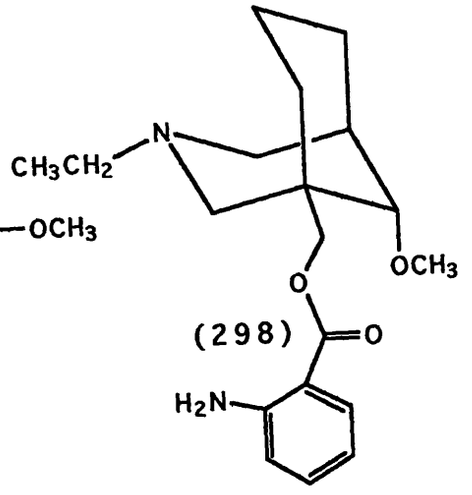

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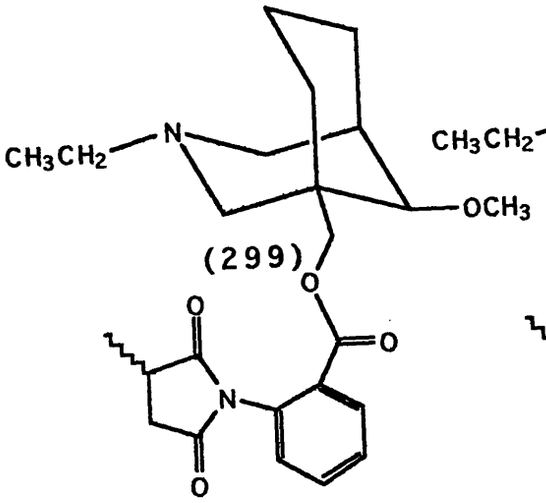
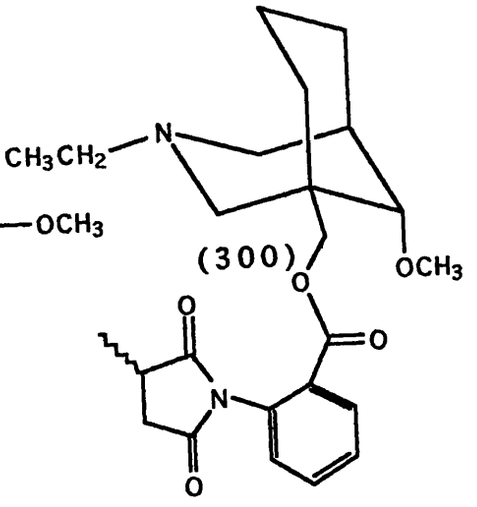


(299)



(300)

d, *J* 9.4, 4_{eq}-H), 2.89-2.82 (1H, m, 7_{ax}-H), 2.56 (1H, dt, *J* 9.4 and 0.9, 4_{ax}-H), 2.52-2.45 (2H, m, 8_{eq}- and 5-H), 2.47 (2H, q, *J* 7.2, NCH_2 CH_3) 2.27-2.05 (3H, m, 6_{ax}-, 6_{eq}- and 8_{ax}-H), 1.56-1.49 (1H, m, 7_{eq}-H), 1.29 (3H, t, *J* 7.2, $CO_2CH_2CH_3$) and 1.10 (3H, t, *J* 7.2, NCH_2CH_3); δ_C ($CDCl_3$)/ppm (100MHz) 216.1 (C-9), 171.1 ($CO_2CH_2CH_3$), 62.3 (C-2), 58.8 (C-4), 52.3 (NCH_2CH_3), 49.2 ($CO_2CH_2CH_3$), 45.3 (C-5), 41.8 (C-1), 36.8 (C-8), 26.9 (C-6), 20.9 (C-7), 14.1 ($CO_2CH_2CH_3$) and 12.7 (NCH_2CH_3); $C_{13}H_{21}NO_3$ requires MW 239 and C, 65.2; H, 8.8; N, 5.8%. Found: m/z (EI) 239 (M^+, 15%), 222 [$(M-OH)^+$, 100%] and C, 65.2; H, 8.9; N, 5.9%. [See Sections 4.2.2.3 and 4.2.3.4]

4.3.2.26 Ethyl 3-ethyl-3-aza-9-(*RS*)-hydroxy-bicyclo[3.3.1]nonane-1-carboxylate (293)

To a stirred solution of ketoester (292) (2.39g, 10.0mmol) in anhydrous ethanol ($25cm^3$) at 5°C (ice-bath) was added dropwise (30min) a suspension of sodium borohydride (224mg, 5.9mmol) in anhydrous ethanol ($20cm^3$) such that the internal temperature did not rise above 5°C. When the addition was complete (45min), the reaction mixture was brought to 25°C (45min) and the stirring continued for 2h. To the resulting solution was added aqueous hydrochloric acid solution (2M, $10cm^3$) and then the acidic solution was basified to pH 8 (saturated aqueous sodium hydrogen carbonate solution then 2M aqueous sodium hydroxide solution). The resulting solution was diluted with water ($75cm^3$) and then extracted with ethyl acetate (3 x $100cm^3$). The combined organic layers were washed with brine ($75cm^3$), dried ($MgSO_4$) and concentrated under reduced pressure to give the essentially pure 9-(*RS*)-hydroxyester (293) as a colourless oil (1.95g, 81%) (mixture of epimers at C-9 a and b, 1:1). TLC (diethyl ether-hexane 1:1, R_f = 0.30 and 0.25); υ_{max} (film)/cm^{-1}: 3458m, 2916s, 1713s, 1453m, 1259s, 1149m and 1070s; Fast eluting epimer (293a): δ_H ($CDCl_3$)/ppm (270MHz) 4.19-4.11 (2H, m, CO_2CH_2 CH_3), 3.93 (1H, d, *J* 2.9, 9_{eq}-H), 3.50-3.42 (1H, m, OH_{ax}), 2.84 (1H, d, *J* 11.9, 2_{eq}-H), 2.71-2.53

(4H, m, 4_{eq}-, 4_{ax}-, 2_{ax}- and 7_{ax}-H), 2.34-2.17 (2H, m, NC*H*$_2$ CH_3), 2.13-1.93 (2H, m, 6_{eq}- and 8_{eq}-H), 1.81-1.64 (2H, m, 5- and 8_{ax}-H), 1.50-1.39 (2H, m, 7_{eq}- and 6_{ax}-H), 1.26 (3H, t, *J* 7.2, $CO_2CH_2CH_3$) and 1.10-0.99 (3H, m, NCH_2CH_3); Slow eluting epimer (293b): δ_H ($CDCl_3$)/ppm (270MHz) 4.19-4.11 (2H, m, CO_2CH_2 CH_3), 3.88 (1H, d, *J* 3.6, 9_{ax}-H), 3.50-3.42 (1H, m, OH_{eq}), 3.16 (1H, d, *J* 11.6, 2_{eq}-H), 2.95 (1H, d, *J* 10.9, 4_{eq}-H), 2.71-2.53 (1H, m, 7_{ax}-H), 2.34-2.17 (3H, m, NC*H*$_2$ CH_3 and 4_{ax}-H), 2.13-1.93 (3H, m, 2_{ax}-, 6_{ax}- and 8_{ax}-H), 1.81-1.64 (2H, m, 5- and 8_{eq}-H), 1.50-1.39 (2H, m, 7_{eq}- and 6_{eq}-H), 1.26 (3H, t, *J* 7.2, $CO_2CH_2CH_3$) and 1.10-0.99 (3H, m, NCH_2CH_3); $C_{13}H_{23}NO_3$ requires MW 241. Found: m/z (EI) 241 (M^+, 10%), 197 [$(MH-OCH_2CH_3)^+$, 100%].

[See Section 4.2.2.3]

4.3.2.27 Ethyl 3-ethyl-3-aza-9-(*R*)-methoxy-bicyclo[3.3.1]nonane-1-carboxylate (294) and ethyl 3-ethyl-3-aza-9-(*S*)-methoxy-bicyclo[3.3.1]nonane-1-carboxylate (295)

To a stirred solution of secondary alcohol (293) (mixture of epimers a and b, ~1:1) (1.81g, 7.5mmol) in anhydrous *N,N*-dimethylformamide (20cm^3) at 25°C was added 60% sodium hydride (dispersion in mineral oil) (330mg, 8.2mmol). The resulting mixture was stirred at 25°C, under an atmosphere of anhydrous nitrogen, for 3h after which methyl iodide (1.17g, 8.2mmol) was added in one portion. The mixture was stirred until completion of the reaction (2h). To the resulting mixture was added aqueous hydrochloric acid solution (2M, 75cm^3) and then the acidic solution was extracted with ethyl acetate (2 x 100cm^3) and then basified to pH 9 (solid sodium hydrogen carbonate then 2M aqueous sodium hydroxide solution). The resulting solution was extracted with ethyl acetate (3 x 100cm^3) and the combined organic layers were washed with brine (75cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The pale yellow oil was purified over silica gel (125g) eluted with diethyl ether-hexane (1:2) to give the less polar epimer a (294) (96mg, 5%) separated from the more polar epimer b (295) (134mg, 7%) plus a mixture of (294) and (295) (917mg,

48%) (a:b, 7:5 respectively). Fast eluting epimer (294): TLC (diethyl ether, R_f = 0.3); υ_{max} (film)/cm^{-1}: 2929s, 1731s, 1455w, 1260s, 1237m and 1106s; δ_H ($CDCl_3$)/ppm (270MHz) 4.20-4.04 (2H, m, CO_2CH_2 CH_3), 3.53 (1H, d, *J* 3.6, 9_{eq}-H), 3.31 (3H, s, OCH_3), 3.01 (1H, dd, *J* 10.9 and 1.0, 2_{eq}-H), 2.91 (1H, dd, *J* 10.9 and 1.0, 2_{ax}-H), 2.65-2.46 (1H, m, 7_{ax}-H), 2.29-2.01 (6H, m, 4_{ax}-, 4_{eq}-, 8_{ax}-, 6_{ax}-H and NCH_2 CH_3), 1.84-1.77 (2H, m, 5- and 8_{eq}-H) 1.48-1.42 (2H, m, 6_{eq} and 7_{eq}-H), 1.25 (3H, t, *J* 7.2, $CO_2CH_2CH_3$) and 1.02 (3H, t, *J* 7.2, NCH_2CH_3); $C_{14}H_{25}NO_3$ requires MW 255. Found: m/z (EI) 255 (M^+, 45%), 224 [$(M-OCH_3)^+$, 100%]. Slow eluting epimer (295): TLC (diethyl ether, R_f = 0.2); υ_{max} (film)/cm^{-1}: 2928s, 1731s, 1453w, 1367w, 1259s, 1104s and 1055m; δ_H ($CDCl_3$)/ppm (270MHz) 4.22-4.03 (2H, m, CO_2CH_2 CH_3), 3.49 (1H, d, *J* 3.6, $9\text{-}H_{ax}$), 3.31 (3H, s, OCH_3), 3.08-2.98 (1H, m, 4_{eq}-H), 2.91 (1H, d, *J* 11.6, 2_{eq}-H), 2.68-2.50 (1H, m, 7_{ax}-H), 2.38-1.43 (10H, m, 4_{ax}-, 2_{ax}-, 8_{ax}-, 5-, 6_{ax}-, 6_{eq}-, 8_{eq}-, 7_{eq}-H and NCH_2 CH_3), 1.25 (3H, t, *J* 7.2, $CO_2CH_2CH_3$) and 1.10-1.02 (3H, m, NCH_2CH_3); $C_{14}H_{25}NO_3$ requires MW 255. Found: m/z (EI) 255 (M^+, 60%), 240 [$(M-CH_3)^+$, 100%]. [See Section 4.2.2.3]

4.3.2.28 3-Ethyl-3-aza-9-(*RS*)-methoxy-bicyclo[3.3.1]nonane-1-methanol (296)

To a stirred solution of methyl ethers (294) and (295) (mixture of epimers a and b, ~7:5 respectively) (1.15g, 4.5mmol) in anhydrous tetrahydrofuran (40cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added dropwise (15min) a suspension of lithium aluminium hydride in anhydrous diethyl ether (4.5cm^3, 1M, 4.5mmol). The resulting suspension was stirred at 25°C until completion of the reaction (2h). Ethyl acetate (5cm^3) and then water (2 x 5cm^3) were cautiously and successively added (CARE-exothermic) to the mixture at 25°C, in order to decompose the excess of hydride after which aqueous hydrochloric acid solution (2M, 6cm^3) was added to the resulting white precipitate. The acidic mixture was basified to pH 8 (saturated aqueous sodium hydrogen carbonate solution 60cm^3) and the resulting mixture was extracted

with ethyl acetate (2 x 100cm^3). The combined organic layers were washed with brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual colourless oil was purified over silica gel (40g) eluted with ethyl acetate to give the essentially pure 9-(*RS*)-methoxy substituted alcohol (296) as a colourless oil (0.92g, 96%) (mixture of epimers at C-9 a and b, 6:5 respectively). TLC (ethyl acetate-hexane 2:1, $R_f = 0.2$); υ_{max} (film)/cm^{-1}: 3422m, 2914s, 1452m and 1104s; δ_H ($CDCl_3$)/ppm (270MHz) 3.50 and 3.42 (1H, 2 x d, *J* 10.9, 9-H), 3.35 and 3.33 (3H, 2 x s, OCH_3), 3.30-3.19 (2H, m, CH_2 OH), 3.02 (1H, br d, *J* 9.4, 4_{eq}-H), 2.73-2.51 (3H, m, 7_{ax}-, 2_{eq}- and OH), 2.28-2.18 (3H, m, 4_{ax}-, 2_{ax}- and 8_{ax}-H), 1.98-1.91 (3H, m, NCH_2 CH_3 and 6_{ax}-H) 1.80-1.70 (1H, m, 5-H), 1.59-1.41 (2H, m, 8_{eq}- and 6_{eq}-H), 1.33-1.30 (1H, m, 7_{eq}-H) and 1.03 (3H, t, *J* 7.2, NCH_2CH_3); $C_{12}H_{23}NO_2$ requires MW 213. Found: m/z (EI) 213 (M^+, 30%), 198 [$(M-CH_3)^+$, 100%].

[See Section 4.2.2.3]

4.3.2.29 3-Ethyl-3-aza-9-(*R*)-methoxy-bicyclo[3.3.1]nonane-1-methyl 2-aminobenzoate (297) and 3-ethyl-3-aza-9-(*S*)-methoxy-bicyclo[3.3.1]nonane-1-methyl 2-aminobenzoate (298)

To a stirred solution of substituted primary alcohol (296) (mixture of epimers a and b, ~6:5 respectively) (0.75g, 3.5mmol, 1.1 equiv.) in anhydrous *N,N*-dimethylformamide (6cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added isatoic anhydride (248) (0.52g, 3.2mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (0.04g, 0.3mmol, 0.1 equiv.) in one portion. The reaction mixture was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (5h). The mixture was cooled to 25°C (45min) and then partitioned between ethyl acetate (25cm^3) and water (25cm^3). The aqueous layer was extracted with ethyl acetate (3 x 25cm^3) and the combined organic layers were then washed successively with water (3 x 50cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual brown oil was purified over silica gel (50g) eluted with ethyl

acetate-hexane (1:4) to give the less polar epimer a (297) (176mg, 17%) separated from the more polar epimer b (298) (171mg, 16%). Fast eluting epimer (297): TLC (ethyl acetate-hexane 4:1, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 3477s, 1680s, 1619s, 1454w, 1295m, 1245m, 1161m, 1103m and 750s; δ_H ($CDCl_3$)/ppm (270MHz) 7.86 (1H, d, *J* 8.0, 6'-H), 7.25 (1H, t, *J* 7.6, 4'-H), 6.67-6.61 (2H, m, 3'- and 5'-H), 5.73 (2H, br s, NH_2), 4.06-4.03 (2H, m, $ArCO_2CH_2$ R), 3.31 (3H, s, OCH_3), 3.15 (1H, d, *J* 2.9, 9-H_{eq}), 3.04 (1H, d, *J* 10.2, 4_{eq}-H), 2.92 (1H, d, *J* 10.8, 2_{eq}-H), 2.67-2.53 (1H, m, 7_{ax}-H), 2.23 (2H, q, *J* 7.2, NCH_2 CH_3), 2.16-2.08 (3H, m, 4_{ax}-, 2_{ax}- and 5-H), 1.91-1.68 (2H, m, 8_{ax}- and 6_{ax}-H), 1.56-1.39 (3H, m, 6_{eq}-, 7_{eq}- and 8_{eq}-H) and 1.02 (3H, t, *J* 7.2, NCH_2CH_3); $C_{19}H_{28}N_2O_3$ requires MW 332. Found: m/z (EI) 332 (M^+, 20%), 317 [$(M-CH_3)^+$, 100%]. Slow eluting epimer (298): TLC (ethyl acetate-hexane 4:1, $R_f = 0.2$); υ_{max} (film)/cm^{-1}: 3463s, 1685s, 1619s, 1442w, 1388m, 1245s, 1137m and 1102s; δ_H ($CDCl_3$)/ppm (270MHz) 7.87 (1H, d, *J* 6.5, 6'-H), 7.26 (1H, t, *J* 6.9, 4'-H), 6.68-6.65 (2H, m, 3'- and 5'-H), 5.74 (2H, br s, NH_2), 4.08-4.05 (2H, m, $ArCO_2CH_2$ R), 3.44-3.22 (4H, m, incorporating δ3.33, 3H, s, OCH_3 and 9-H_{ax}), 3.16 (1H, d, *J* 9.4, 2_{eq}-H), 3.01 (1H, d, *J* 10.7, 4_{eq}-H), 2.74-2.52 (2H, m, 7_{ax}- and 2_{ax}-H), 1.42 (1H, d, *J* 10.7, 4_{ax}-H), 2.32-2.17 (3H, m, 5-H and NCH_2 CH_3), 2.10-2.03 (2H, m, 6_{ax}- and 8_{ax}-H), 1.93-1.74 (2H, m, 6_{eq}- and 8_{eq}-H), 1.48-1.38 (1H, m, 7_{eq}-H) and 1.02 (3H, t, *J* 7.2, NCH_2CH_3); $C_{19}H_{28}N_2O_3$ requires MW 332. Found: m/z (EI) 332 (M^+, 15%), 317 [$(M-CH_3)^+$, 100%].

[See Sections 4.2.2.1, 4.2.3.1 and 4.2.3.4]

4.3.2.30 3-Ethyl-3-aza-9-(*R*)-methoxy-bicyclo[3.3.1]nonane-1-methyl [2-(*RS*)-methylsuccinimido]benzoate (299)

A mixture of 2-aminobenzoate (297) (100mg, 0.3mmol) and (*RS*)-methylsuccinic anhydride (257) (69mg, 0.6mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (3h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate ($10cm^3$) and saturated aqueous sodium hydrogen carbonate solution ($10cm^3$). The aqueous layer was extracted with

ethyl acetate (3 x 10cm^3) and the combined organic layers were washed successively with saturated aqueous sodium hydrogen carbonate solution (2 x 20cm^3) and brine (15cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual oil was purified over silica gel (12g) eluted with ethyl acetate-hexane (1:1) to give the [2-(*RS*)-methylsuccinimido]benzoate (299) as a colourless oil (88mg, 69%). TLC (ethyl acetate, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 2928m, 1715s, 1603w, 1492m, 1453m, 1390m, 1262m, 1189m, 1102w and 746m; δ_H ($CDCl_3$)/ppm (270MHz) 8.10 (1H, br d, *J* 4.4, 6'-H), 7.67 (1H, t, *J* 7.0, 4'-H), 7.54 (1H, t, *J* 7.0, 5'-H), 7.26 (1H, d, *J* 7.0, 3'-H), 4.11-4.01 (2H, m, $ArCO_2CH_2$ R), 3.44-2.89 (8H, m incorporating δ3.29, 3H, s, OCH_3 and δ2.89, 1H, d, *J* 11.6, 2_{eq}-H, plus 4_{eq}-, 9_{eq}-, 2"- and 3"-H), 2.61-2.48 (2H, m, 7_{ax}- and 3"-H), 2.24-2.05 (5H, m, 4_{ax}-, 2_{ax}-, 5-H and NCH_2 CH_3) 1.82-1.70 (2H, m, 6_{ax}- and 8_{ax}-H), 1.50-1.45 (6H, m, 6_{eq}-, 7_{eq}-, 8_{eq}-H and 5"-H_3) and 1.03 (3H, br t, *J* 7.2, NCH_2CH_3); δ_C ($CDCl_3$)/ppm (100MHz) 179.9 (C-1"), 176.1 (C-4"), 163.8 (OC=O), 133.4 (C-4'), 132.9 (C-2'), 131.4 (C-6'), 129.9 (C-3'), 129.4 (C-5'), 127.6 (C-1'), 81.0 (C-9), 70.1 ($ArCO_2CH_2R$), 61.3 (C-2), 58.3 (C-4), 55.9 (OCH_3), 52.3 (NCH_2CH_3), 38.3 (C-1), 37.0 (C-3"), 35.4 and 35.2 (C-2", both isomers), 30.6 (C-5), 28.0 (C-8), 24.3 (C-6), 20.5 (C-7), 16.5 and 16.3 (C-5", both isomers) and 12.8 (NCH_2CH_3); $C_{24}H_{32}N_2O_5$ requires MW 428 and C, 67.3; H, 7.5; N, 6.5%. Found: m/z (EI) 428 (M^+, 25%), 72 {$[CH_3CH_2N(CH_3)=CH_2]^+$, 100%} and C, 67.1; H, 7.6; N, 6.5%.

[See Sections 4.2.2.2, 4.2.3.2 and 4.2.3.4]

4.3.2.31 3-Ethyl-3-aza-9-(*S*)-methoxy-bicyclo[3.3.1]nonane-1-methyl [2-(*RS*)-methylsuccinimido]benzoate (300)

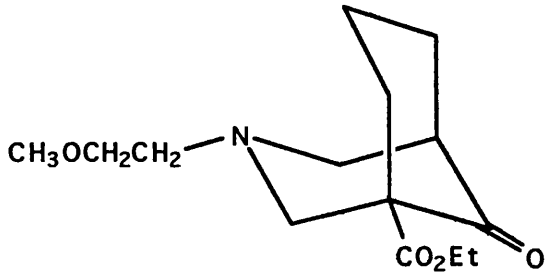
A mixture of 2-aminobenzoate (298) (133mg, 0.4mmol) and (*RS*)-methylsuccinic anhydride (257) (91mg, 0.8mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (4h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (10cm^3) and saturated aqueous sodium hydrogen carbonate solution (10cm^3). The aqueous layer was extracted with

ethyl acetate (3 x 10cm^3) and the combined organic layers were washed successively with saturated aqueous sodium hydrogen carbonate solution (2 x 20cm^3) and brine (15cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual oil was purified over silica gel (12g) eluted with ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (300) as a colourless oil (107mg, 62%). TLC (ethyl acetate, $R_f = 0.2$); υ_{max} (film)/cm^{-1}: 2929m, 1717s, 1603w, 1492m, 1453m, 1391m, 1261s, 1188m, 1138m, 1100s and 745m; δ_H ($CDCl_3$)/ppm (270MHz) 8.12 (1H, d, *J* 8.0, 6'-H), 7.66 (1H, t, *J* 7.2, 4'-H), 7.53 (1H, t, *J* 7.0, 5'-H), 7.25 (1H, d, *J* 7.1, 3'-H), 4.06-3.96 (2H, m, $ArCO_2CH_2$ R), 3.30 (3H, s, OCH_3) 3.16-3.05 (3H, m, 9_{ax}-, 2"- and 3"-H), 2.75-2.59 (4H, m, 2_{eq}-, 4_{eq}-, 7_{ax}- and 3"-H), 2.42-2.21 (4H, m, 4_{ax}-, 2_{ax}-H and NCH_2 CH_3), 2.09-2.04 (2H, m, 6_{ax}- and 5-H), 1.92-1.80 (1H, m, 8_{ax}- or 8_{eq}-H), 1.67-1.38 (6H, m, 6_{eq}-, 7_{eq}-, 8_{eq}- or 8_{ax}-H and 5"-H_3) and 1.05 (3H, br t, *J* 7.2, NCH_2CH_3); δ_C ($CDCl_3$)/ppm (100MHz) 179.9 (C-1"), 176.1 (C-4"), 164.1 (OC=O), 133.3 (C-4'), 132.9 (C-2'), 131.3 (C-6'), 129.9 (C-3'), 129.3 (C-5'), 127.6 (C-1'), 81.0 (C-9), 70.2 ($ArCO_2CH_2R$), 61.3 (C-2), 60.4 (C-4), 55.6 (OCH_3), 52.2 (NCH_2CH_3), 39.1 (C-1), 37.0 (C-3"), 35.4 and 35.2 (C-2", both isomers), 31.3 (C-5), 30.3 (C-8), 28.9 (C-6), 21.1 (C-7), 16.5 and 16.3 (C-5", both isomers) and 12.6 (NCH_2CH_3); $C_{24}H_{32}N_2O_5$ requires MW 428 and C, 67.3; H, 7.5; N, 6.5%. Found: m/z (EI) 428 (M^+, 30%), 413 [$(M-CH_3)^+$, 100%] and C, 67.4; H, 7.4; N, 6.4%.

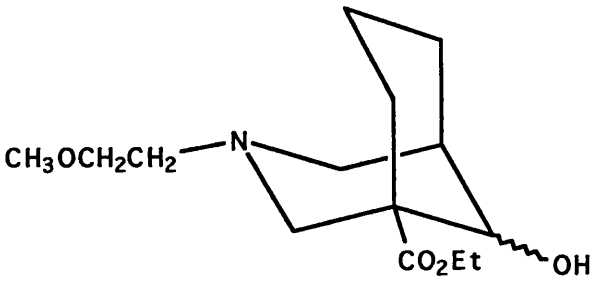
[See Sections 4.2.2.2, 4.2.3.2 and 4.2.3.4]

4.3.2.32 Ethyl 3-(2-methoxyethyl)-3-aza-bicyclo[3.3.1]nonan-9-one-1-carboxylate (302)

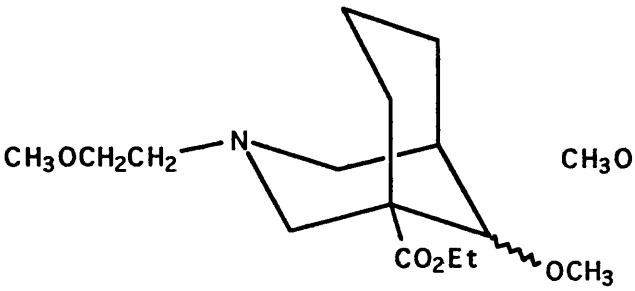
To a stirred solution of 2-methoxyethylamine (301) (13.3cm^3, 150mmol) in ethanol (35cm^3) at 25°C was added dropwise (10min) 37% aqueous formaldehyde (24.3cm^3, 300mmol, 2.0 equiv.). The resulting solution was added in one portion to a stirred solution of ethyl 2-cyclohexanone-1-carboxylate (291) (26.8g, 150mmol) in ethanol (85cm^3) at 25°C. Glacial acetic



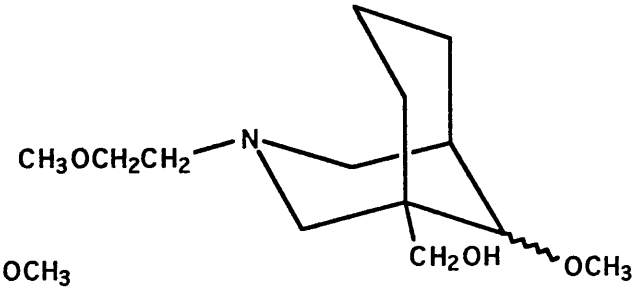
(302)



(303)



(304)



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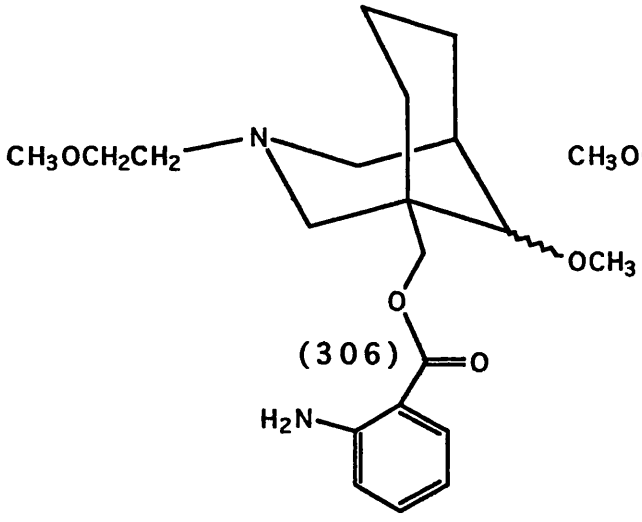
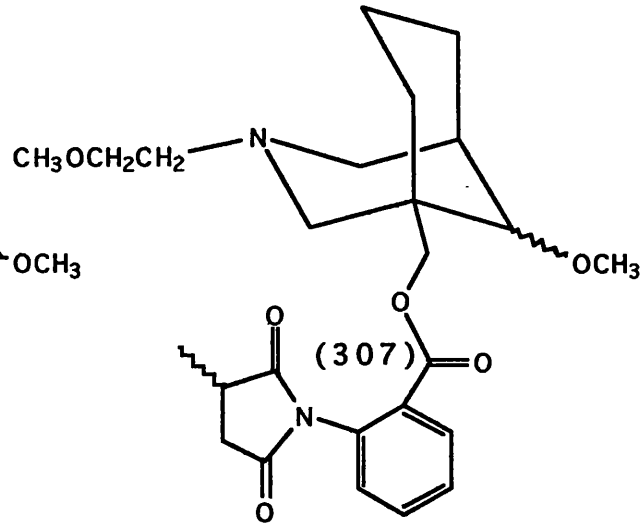


(306)



(307)

acid (1.5cm^3) was added to the solution which was then heated under reflux (oil-bath), under an atmosphere of nitrogen, and the stirring continued until completion of the reaction (4h). The reaction mixture was cooled to 25°C (17h), concentrated under reduced pressure and then diluted with ethyl acetate (250cm^3). The organic layer was washed successively with saturated aqueous sodium hydrogen carbonate solution (160cm^3) and aqueous hydrochloric acid solution (2M, 2 x 160cm^3). The combined acidic layers were extracted with ethyl acetate (120cm^3) and then basified to pH 9 (solid sodium hydrogen carbonate then 2M aqueous sodium hydroxide solution). The mixture was then extracted with ethyl acetate (2 x 160cm^3) and the combined organic layers were washed with brine (2 x 85cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual orange oil was purified by distillation under reduced pressure (Bp 142-144°C/0.15mmHg) to give the ketoester (302) as a colourless oil (27.4g, 68%). TLC (ethyl acetate, $R_f = 0.4$); υ_{max} (film)/cm^{-1}: 2930m, 2820m, 1735s, 1730s, 1455w, 1365w, 1255s, 1100s and 1055w; δ_H ($CDCl_3$)/ppm (270MHz) 4.21 (2H, q, *J* 7.1, CO_2CH_2 CH_3), 3.54 (2H, t, *J* 5.7, NCH_2CH_2 OCH_3), 3.37 (3H, s, $NCH_2CH_2OCH_3$), 3.24 (1H, dd, *J* 10.9 and 1.0, 2_{eq}-H), 3.17 (1H, dt, *J* 10.9 and 1.0, 4_{eq}-H), 2.92-2.80 (1H, m, 7_{ax}-H), 2.60 (1H, dd, *J* 10.9 and 1.0, 2_{ax}-H), 2.59 (2H, t, *J* 5.7, NCH_2 CH_2OCH_3), 2.54-2.46 (3H, m, 4_{ax}-, 8_{eq}- and 5-H), 2.28-2.07 (3H, m, 6_{ax}-, 6_{eq}- and 8_{ax}-H), 1.84-1.52 (1H, m, 7_{eq}-H) and 1.29 (3H, t, *J* 7.1, $CO_2CH_2CH_3$); δ_C ($CDCl_3$)/ppm (100MHz) 212.4 (C-9), 171.1 ($CO_2CH_2CH_3$), 70.7 ($NCH_2CH_2OCH_3$), 62.3 (C-2), 61.1($CO_2CH_2CH_3$), 60.7 (C-4), 58.9 (C-1), 58.7 ($NCH_2CH_2OCH_3$), 56.2 ($NCH_2CH_2OCH_3$), 47.3 (C-5), 36.8 (C-8), 34.1 (C-6), 20.5 (C-7) and 14.1 ($CO_2CH_2CH_3$); $C_{14}H_{23}NO_4$ requires MW 269 and C, 62.4; H, 8.6; N, 5.2%. Found: m/z (EI) 269 (M^+, 5%), 224 [$(M-OCH_2CH_3)^+$, 100%] and C, 62.3; H, 8.5; N, 5.1%. [See Sections 4.2.2.3 and 4.2.3.4]

4.3.2.33 Ethyl 3-(2-methoxyethyl)-3-aza-9-(*RS*)-hydroxy-bicyclo[3.3.1]nonane-1-carboxylate (303)

To a stirred solution of ketoester (302) (2.69g, 10.0mmol) in anhydrous ethanol ($25cm^3$) at 5°C (ice-bath) was added dropwise (30min) a suspension of sodium borohydride (224mg, 5.9mmol) in anhydrous ethanol ($20cm^3$) such that the internal temperature did not rise above 5°C. When the addition was complete (45min), the reaction mixture was brought to 25°C (30min) and the stirring continued for 3h. To the resulting solution was added aqueous hydrochloric acid solution (2M, $10cm^3$) and then the acidic solution was basified to pH 8 (saturated aqueous sodium hydrogen carbonate solution then 2M aqueous sodium hydroxide solution). The resulting solution was diluted with water ($75cm^3$) and then extracted with ethyl acetate (3 x $100cm^3$). The combined organic layers were washed with brine ($75cm^3$), dried ($MgSO_4$) and concentrated under reduced pressure to give the essentially pure 9-(*RS*)-hydroxyester (303) as a colourless oil (1.78g, 66%) (mixture of epimers at C-9 a and b, 1:1). TLC (diethyl ether-hexane 2:1, $R_f = 0.30$ and 0.25); υ_{max} (film)/cm^{-1}: 3455m, 2914s, 2870m, 2825m, 1710s, 1456m, 1261s, 1145w and 1072s; Fast eluting epimer (303a): δ_H ($CDCl_3$)/ppm (270MHz) 4.20-4.12 (2H, m, CO_2CH_2 CH_3), 3.95 (1H, d, *J* 2.9, 9_{eq}-H), 3.52-3.48 (2H, m, NCH_2CH_2 OCH_3), 3.48-3.40 (1H, m, OH_{ax}), 3.36 (3H, s, $NCH_2CH_2OCH_3$), 3.02-2.96 (1H, m, 4_{eq}-H), 2.88 (1H, d, *J* 11.6, 2_{eq}-H), 2.70-2.51 (2H, m, 4_{ax}- and 7_{ax}-H), 2.43-2.25 (3H, m, 2_{ax}-H and NCH_2 CH_2OCH_3), 2.15-1.94 (2H, m, 6_{eq}- and 8_{eq}-H), 1.80-1.65 (2H, m, 5- and 8_{ax}-H), 1.53-1.41 (2H, m, 7_{eq}- and 6_{ax}-H) and 1.25 (3H, t, *J* 7.2, $CO_2CH_2CH_3$); Slow eluting epimer (303b): δ_H ($CDCl_3$)/ppm (270MHz) 4.20-4.12 (2H, m, CO_2CH_2 CH_3), 3.87 (1H, d, *J* 3.6, 9_{ax}-H), 3.52-3.48 (2H, m, NCH_2CH_2 OCH_3), 3.48-3.40 (1H, m, OH_{eq}), 3.36 (3H, s, $NCH_2CH_2OCH_3$), 3.02-2.96 (1H, m, 4_{eq}-H), 2.70-2.51 (3H, m, 2_{eq}-, 4_{ax}- and 7_{ax}-H), 2.43-2.25 (4H, m, 4_{ax}-, 2_{ax}-H and NCH_2 CH_2OCH_3), 2.15-1.94 (3H, m, 2_{ax}-, 6_{ax}- and 8_{ax}-H), 1.80-1.65 (2H, m, 5- and 8_{eq}-H), 1.53-1.41 (2H, m, 7_{eq}- and 6_{eq}-H) and 1.25 (3H, t, *J* 7.2, $CO_2CH_2CH_3$); $C_{14}H_{25}NO_4$ requires MW 271. Found: m/z (EI) 271 (M^+, 15%), 227 [$(MH\text{-}OCH_2CH_3)^+$, 100%]. [See Section 4.2.2.3]

4.3.2.34 Ethyl 3-(2-methoxyethyl)-3-aza-9-(*RS*)-methoxy-bicyclo[3.3.1]nonane-1-carboxylate (304)

To a stirred solution of secondary alcohol (303) (mixture of epimers a and b, ~1:1) (2.03g, 7.5mmol) in anhydrous *N,N*-dimethylformamide (20cm^3) at 25°C was added 60% sodium hydride (dispersion in mineral oil) (330mg, 8.2mmol). The resulting mixture was stirred at 25°C, under an atmosphere of nitrogen, for 2h after which methyl iodide (1.17g, 8.2mmol) was added in one portion. The mixture was stirred until completion of the reaction (2h). To the resulting mixture was added aqueous hydrochloric acid solution (2M, 75cm^3) and then the acidic solution was extracted with ethyl acetate (2 x 100cm^3) and then basified to pH 9 (solid sodium hydrogen carbonate then 2M aqueous sodium hydroxide solution). The resulting solution was extracted with ethyl acetate (3 x 100cm^3) and the combined organic layers were washed with brine (75cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The pale yellow oil was purified over silica gel (125g) eluted with ethyl acetate-hexane (2:1) to give (304) (0.53g, 25%) (mixture of epimers at C-9 a and b, 8:5 respectively). TLC (ethyl acetate-hexane 2:1, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 2929s, 2861m, 2821m, 1731s, 1169s, 1453w, 1367w, 1257s, 1104s and 1053w; Fast eluting epimer (304a): δ_H ($CDCl_3$)/ppm (270MHz) 4.20-4.04 (2H, m, CO_2CH_2 CH_3), 3.55-3.46 (3H, m, 9_{eq}-H and NCH_2CH_2 OCH_3), 3.35 (3H, s, $NCH_2CH_2OCH_3$), 3.33 (3H, s, OCH_{3ax}), 3.25-3.11 (1H, m, 4_{eq}-H), 2.98-2.88 (1H, m, 2_{eq}-H), 2.71-2.57 (1H, m, 4_{ax}), 2.66-2.53 (1H, m, 7_{ax}-H), 2.37-1.46 (9H, m, 2_{ax}-, 8_{ax}-, 5-, 6_{ax}-, 6_{eq}-, 8_{eq}-, 7_{eq}-H and NCH_2 CH_2OCH_3) and 1.27 (3H, t, *J* 7.2, $CO_2CH_2CH_3$); Slow eluting epimer (304b): δ_H ($CDCl_3$)/ppm (270MHz) 4.20-4.04 (2H, m, CO_2CH_2 CH_3), 3.55-3.46 (3H, m, 9_{ax}-H and NCH_2CH_2 OCH_3), 3.35 (3H, s, $NCH_2CH_2OCH_3$), 3.33 (3H, s, OCH_{3eq}), 2.98-2.88 (1H, m, 4_{eq}-H), 2.71-2.57 (1H, m, 4_{ax}), 2.66-2.53 (1H, m, 7_{ax}-H), 2.37-1.46 (10H, m, 2_{eq}-, 2_{ax}-, 8_{ax}-, 5-, 6_{ax}-, 6_{eq}-, 8_{eq}-, 7_{eq}-H and NCH_2 CH_2OCH_3) and 1.27 (3H, t, *J* 7.2, $CO_2CH_2CH_3$); $C_{15}H_{27}NO_4$ requires MW 285. Found: m/z (EI) 285 (M^+, 40%), 270 [$(M-CH_3)^+$, 100%]. [See Section 4.2.2.3]

4.3.2.35 3-(2-Methoxyethyl)-3-aza-9-(*RS*)-methoxy-bicyclo[3.3.1]nonane-1-methanol (305)

To a stirred solution of methyl ethers (304) (mixture of epimers a and b, ~8:5 respectively) (1.00g, 3.5mmol) in anhydrous tetrahydrofuran (30cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added dropwise (15min) a suspension of lithium aluminium hydride in anhydrous diethyl ether (3.5cm^3, 1M, 3.5mmol). The resulting suspension was stirred at 25°C until completion of the reaction (1h). Ethyl acetate (4cm^3) and then water (2 x 4cm^3) were cautiously and successively added (CARE-exothermic) to the mixture at 25°C, in order to decompose the excess of hydride after which aqueous hydrochloric acid solution (2M, 5cm^3) was added to the resulting white precipitate. The acidic mixture was basified to pH 8 (saturated aqueous sodium hydrogen carbonate solution 45cm^3) and the resulting mixture was extracted with ethyl acetate (2 x 70cm^3). The combined organic layers were washed with brine (60cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual colourless oil was purified over silica gel (40g) eluted with ethyl acetate to give the essentially pure 9-(*RS*)-methoxy substituted alcohol (305) as a colourless oil (0.75g, 89%) (mixture of epimers at C-9 a and b, 4:3 respectively). TLC (ethyl acetate-hexane 4:1, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 3424s, 2917s, 2861m, 2842m, 1452m, 1173s and 1102s; δ_H ($CDCl_3$)/ppm (270MHz) 3.52 and 3.40 (1H, 2 x d, *J* 10.9, 9-H), 3.36, 3.35 and 3.32 (6H, 3 x s, OCH_3 and $NCH_2CH_2OCH_3$), 3.30-3.91 (4H, m, 4_{eq}-, 2_{eq}-H and CH_2 OH), 2.71-2.50 (5H, m, OH, 4_{ax}-, 7_{ax}-H and NCH_2CH_2 OCH_3), 2.28-2.12 (4H, m, 2_{ax}-, 8_{ax}-H and NCH_2 CH_2OCH_3), 1.96-1.90 (1H, m, 6_{ax}-H) 1.78-1.71 (1H, m, 5-H), 1.60-1.44 (2H, m, 8_{eq}- and 6_{eq}-H) and 1.35-1.30 (1H, m, 7_{eq}-H); $C_{13}H_{25}NO_3$ requires MW 243. Found: m/z (EI) 243 (M^+, 15%), 228 [$(M-CH_3)^+$, 100%].

[See Section 4.2.2.3]

4.3.2.36 3-(2-Methoxyethyl)-3-aza-9-(*R,S*)-methoxy-bicyclo[3.3.1]nonane-1-methyl 2-aminobenzoate (306)

To a stirred solution of substituted primary alcohol (305) (mixture of epimers a and b, ~4:3 respectively) (0.51g, 2.1mmol, 1.1 equiv.) in anhydrous *N,N*-dimethylformamide (4cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added isatoic anhydride (248) (0.32g, 1.9mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (24mg, 0.2mmol, 0.1 equiv.) in one portion. The reaction mixture was then heated to 60°C (oil-bath) and the stirring continued until completion of the reaction (6h). The mixture was cooled to 25°C (45min) and then partitioned between ethyl acetate (15cm^3) and water (15cm^3). The aqueous layer was extracted with ethyl acetate (3 x 15cm^3) and the combined organic layers were then washed successively with water (3 x 30cm^3) and brine (25cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual brown oil was purified over silica gel (30g) eluted with ethyl acetate-hexane (1:4) to give (306) (0.29g, 38%) (mixture of epimers at C-9). TLC (ethyl acetate-hexane 4:1, $R_f = 0.2$); υ_{max} (film)/cm^{-1}: 3480s, 2846m, 2841m, 1680s, 1621s, 1454m, 1298m, 1245w, 1161m, 1100m and 747s; δ_H ($CDCl_3$)/ppm (270MHz) 7.85 (1H, d, *J* 7.8, 6'-H), 7.28 (1H, t, *J* 7.8, 4'-H), 6.69-6.61 (2H, m, 3'- and 5'-H), 5.70 (2H, br s, NH_2), 4.08-4.04 (2H, m, $ArCO_2CH_2$ R), 3.54-3.49 (2H, m, NCH_2CH_2 OCH_3), 3.33 (3H, s, $NCH_2CH_2OCH_3$), 3.30 (3H, s, OCH_3), 3.12 (1H, d, *J* 2.9, 9-H), 3.00-2.91 (2H, m, 2_{eq}- and 4_{eq}-H), 2.65-2.52 (1H, m, 7_{ax}-H), 2.35 (2H, t, *J* 5.6, NCH_2 CH_2OCH_3), 2.29-2.21 (2H, m, 4_{ax}- and 2_{ax}-H), 2.16-2.10 (1H, m, 5-H), 1.89-1.69 (2H, m, 8_{ax}- and 6_{ax}-H) and 1.55-1.40 (3H, m, 6_{eq}-, 7_{eq}- and 8_{eq}-H); $C_{20}H_{30}N_2O_4$ requires MW 362. Found: m/z (EI) 362 (M^+, 40%), 347 [$(M-CH_3)^+$, 100%]. [See Sections 4.2.2.1, 4.2.3.1 and 4.2.3.4]

4.3.2.37 3-(2-Methoxyethyl)-3-aza-9-methoxy-bicyclo[3.3.1]nonane-1-methyl [2-(*RS*)-methylsuccinimido]benzoate (307)

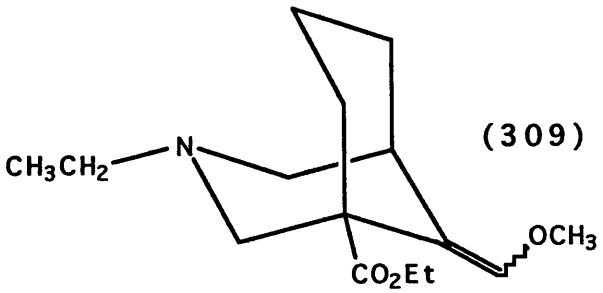
A mixture of 2-aminobenzoate (306) (mixture of epimers) (181mg, 0.50mmol) and (*RS*)-methylsuccinic anhydride (257) (114mg, 1.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (4h). The resulting dark brown gum was cooled to 25°C (20min) and then partitioned between ethyl acetate ($10cm^3$) and saturated aqueous sodium hydrogen carbonate solution ($10cm^3$). The aqueous layer was extracted with ethyl acetate (3 x $10cm^3$) and the combined organic layers were washed successively with saturated aqueous sodium hydrogen carbonate solution (2 x $15cm^3$) and brine ($10cm^3$), dried ($MgSO_4$) and concentrated under reduced pressure. The residual oil was purified over silica gel (12g) eluted with ethyl acetate to give the [2-(*RS*)-methylsuccinimido]benzoate (307) as a colourless oil (112mg, 49%) (mixture of isomers). TLC (ethyl acetate, $R_f = 0.2$); υ_{max} (film)/cm^{-1}: 2938m, 2861m, 2839m, 1715s, 1600m, 1492m, 1453w, 1262m, 1191m, 1102w and 747m; δ_H ($CDCl_3$)/ppm (270MHz) 8.11 (1H, br d, *J* 4.4, 6'-H), 7.68 (1H, t, *J* 7.2, 4'-H), 7.53 (1H, t, *J* 7.2, 5'-H), 7.25 (1H, d, *J* 7.2, 3'-H), 4.12-4.03 (2H, m, $ArCO_2CH_2$ R), 3.54-3.50 (2H, m, NCH_2CH_2 OCH_3), 3.42-2.86 (11H, m incorporating δ3.29, 3H, s, OCH_3 and δ3.28, 3H, s, $NCH_2CH_2OCH_3$, plus 4_{eq}-, 2_{eq}-, 9-, 2"- and 3"-H), 2.62-2.45 (4H, m, 7_{ax}-, 3"-H and NCH_2 CH_2OCH_3), 2.37-2.08 (3H, m, 4_{ax}-, 2_{ax}- and 5-H) 1.81-1.72 (2H, m, 6_{ax}- and 8_{ax}-H) and 1.49-1.44 (6H, m, 6_{eq}-, 7_{eq}-, 8_{eq}-H and 5"-H_3); δ_C ($CDCl_3$)/ppm (100MHz) 179.9 (C-1"), 176.1 (C-4"), 164.1 (OC=O), 133.2 (C-4'), 132.9 (C-2'), 131.5 (C-6'), 129.9 (C-3'), 129.2 (C-5'), 127.5 (C-1'), 81.0 (C-9), 70.2 ($ArCO_2CH_2R$), 61.3 (C-2), 60.4 (C-4), 59.1 ($NCH_2CH_2OCH_3$), 58.2 ($NCH_2CH_2OCH_3$), 55.8 (OCH3), 42.4 ($NCH_2CH_2OCH_3$), 38.7 (C-1), 37.1 (C-3"), 35.5 and 35.2 (C-2", both isomers), 31.0 (C-5), 28.1 (C-8), 24.2 (C-6), 20.5 (C-7) and 16.5 and 16.2 (C-5", both Isomers); $C_{25}H_{34}N_2O_6$ requires MW 458 and C, 65.5; H, 7.5; N, 6.1%. Found: m/z (EI) 458 (M^+, 15%), 102 {$[CH_3OCH_2CH_2N(CH_3)=CH_2]^+$, 100%} and C, 65.3; H, 7.6; N, 6.2%.

[See Sections 4.2.2.2, 4.2.3.2 and 4.2.3.4]

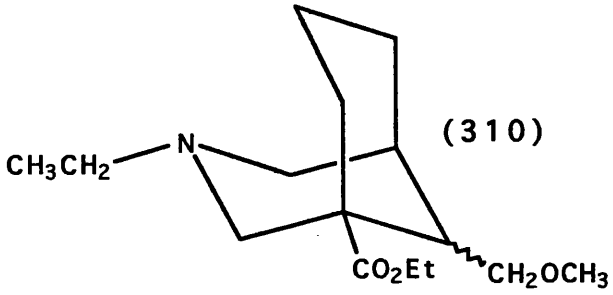
4.3.2.38 Ethyl 3-ethyl-3-aza-9-(*EZ*)-methoxymethylidene-bicyclo[3.3.1]nonane-1-carboxylate (309)

To a stirred suspension of (methoxymethyl)triphenylphosphonium chloride (308) (12.86g, 37.5mmol, 1.5 equiv.) in anhydrous tetrahydrofuran (75cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added ^{n}butyllithium as a solution in hexane (23.4cm^3, 1.6M, 37.5mmol, 1.5 equiv.). After stirring the resulting deep red suspension (30min), a solution of ketoester (292) (5.98g, 25.0mmol) in anhydrous tetrahydrofuran (38cm^3) was then added with stirring over 20min. The stirring was continued until completion of the reaction (16h) and the reaction mixture was then added to saturated aqueous ammonium chloride solution (100cm^3). The aqueous layer was extracted with ethyl acetate (3 x 75cm^3) and the combined organic layers were washed with brine (75cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual dark red oil was then diluted with diethyl ether (28cm^3) and cooled to 5°C (16h). The mixture was then filtered in order to remove the precipitated triphenylphosphine oxide and the residue was washed with cold (5°C) diethyl ether (125cm^3). The filtrate was concentrated under reduced pressure and the residual dark orange oil was purified over silica gel (175g) eluted with diethyl ether-hexane (1:4) to give the (*EZ*)-enol ether (309) as a pale yellow oil (2.80g, 42%) (mixture of *Z* and *E* isomers, 4:3 respectively). TLC (diethyl ether-hexane 1:3, R_f = 0.3 and 0.2); υ_{max} (film)/cm^{-1}: 3023m, 1727s, 1680s, 1471m, 1438m, 1243s and 802m; Fast eluting isomer (309a): δ_H ($CDCl_3$)/ppm (270MHz) 5.77 (1H, s, C*H*aOCH$_3$), 4.11 (2H, q, *J* 7.3, CO_2CH_2 CH_3), 3.47 (3H, s, CHOC*H*$_3$ a), 2.95 (1H, d, *J* 7.8, 2_{eq}-H), 2.90-2.85 (1H, m, 4_{eq}-H), 2.79-2.67 (1H, m, 7_{ax}-H), 2.50-2.31 (3H, m, 2_{ax}-H and NCH_2 CH_3), 2.32-2.28 (2H, m, 4_{ax}- and 8_{ax}-H), 2.15-2.07 (1H, m, 5-H), 1.92-1.68 (3H, m, 8_{eq}-, 6_{eq}- and 6_{ax}-H), 1.60-1.49 (1H, m, 7_{eq}-H), 1.29 (3H, t, *J* 7.3, $CO_2CH_2CH_3$) and 1.05 (3H, t, *J* 7.2, NCH_2CH_3); Slow eluting isomer (309b): δ_H ($CDCl_3$)/ppm (270MHz) 5.70 (1H, s, C*H*bOCH$_3$), 4.21 (2H, q, *J* 7.3, CO_2CH_2 CH_3), 3.53 (3H, s, CHOC*H*$_3$ b), 3.07-3.03 (2H, m, 2_{eq}- and 4_{eq}-H), 3.02-2.97 (2H, m, 7_{ax}- and 5-H), 2.50-2.31 (2H, m, NCH_2 CH_3), 2.15-2.07 (1H, m, 8_{ax}-H), 2.03-1.98 (2H, m, 2_{ax}- and 4_{ax}-H), 1.92-1.68 (3H, m, 8_{eq}-, 6_{eq}- and

$Ph_3P^+CH_2OCH_3\ Cl^-$ (308)



(309)



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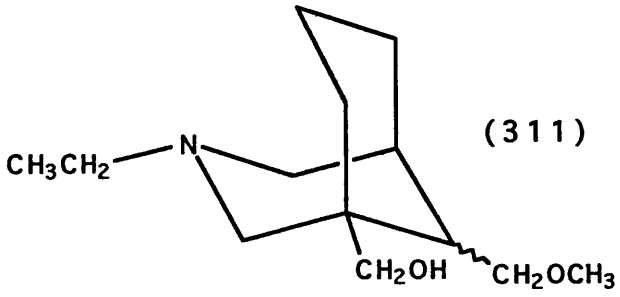

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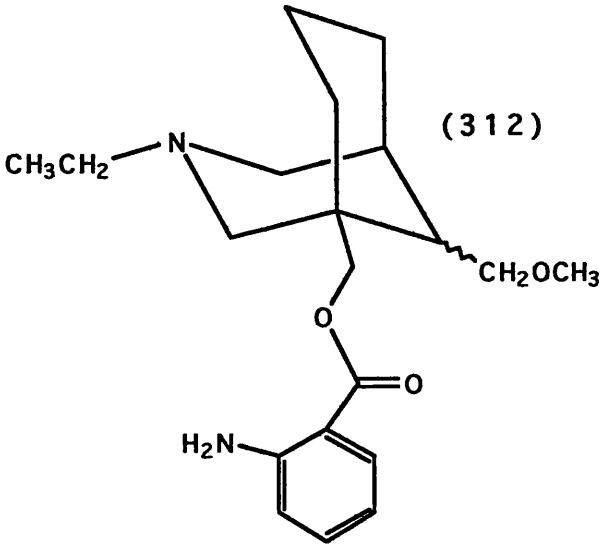

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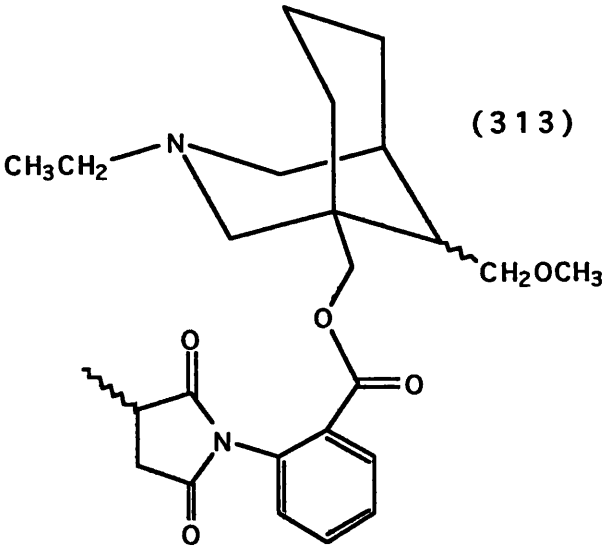


(313)

6_{ax}-H), 1.60-1.49 (1H, m, 7_{eq}-H), 1.29 (3H, t, *J* 7.3, $CO_2CH_2CH_3$) and 1.05 (3H, t, *J* 7.2, NCH_2CH_3); $C_{15}H_{25}NO_3$ requires MW 267. Found: m/z (EI) 267 (M^+, 10%), 222 [$(M-OCH_2CH_3)^+$, 100%]. [See Section 4.2.2.3]

4.3.2.39 Ethyl 3-ethyl-3-aza-9-(*RS*)-methoxymethyl-bicyclo[3.3.1]nonane-1-carboxylate (310)

A solution of enol ether (309) (mixture of *E/Z* geometrical isomers ~4:3 ratio a:b respectively) (2.67g, 10.0mmol) in anhydrous 1,2-dimethoxyethane (35cm^3) was stirred with palladium on charcoal (10%, 3.50g) under hydrogen (5 atm.) at 25°C until completion of the reaction (2h). The mixture was filtered through celite and the residue was washed with diethyl ether (120cm^3). The filtrate was concentrated under reduced pressure and the residual yellow oil was purified over silica gel (150g) eluted with diethyl ether-hexane (4:1) to give the 9-(*RS*)-methoxymethyl ether (310) as a light golden oil (1.69g, 63%) (mixture of epimers at C-9 a and b, 4:3, respectively). TLC (diethyl ether-hexane 1:4, R_f = 0.4 and 0.3); υ_{max} (film)/cm^{-1}: 2917s, 2850m, 1725s, 1462m, 1445m, 1365m, 1121s, 855m and 735w; Fast eluting epimer (310a): δ_H ($CDCl_3$)/ppm (270MHz) 4.14-4.06 (2H, m, CO_2CH_2 CH_3), 3.49-3.40 (2H, m, $CHCH_2$ OCH_{3ax}), 3.33 (3H, s, $CHCH_2OCH_{3\ ax}$), 3.03-2.98 (1H, m, 4_{eq}-H), 2.92-1.89 (1H, m, 2_{eq}-H), 2.65-2.49 (1H, m, 7_{ax}-H), 2.36-2.28 (3H, m, 8_{ax}-H and NCH_2 CH_3), 2.14-2.10 (2H, m, 4_{ax}- and 2_{ax}-H), 2.00-1.55 (6H, m, 5-, 8_{eq}-, 7_{eq}-, 6_{ax}-, 6_{eq}- and 9_{eq}-H), 1.25 (3H, t, *J* 7.2, $CO_2CH_2CH_3$) and 1.05 (3H, t, *J* 7.2, NCH_2CH_3); Slow eluting epimer (310b): δ_H ($CDCl_3$)/ppm (270MHz) 4.14-4.06 (2H, m, CO_2CH_2 CH_3), 3.49-3.40 (2H, m, $CHCH_2$ OCH_{3eq}), 3.36 (3H, s, $CHCH_2OCH_{3\ eq}$), 3.03-2.98 (1H, m, 4_{eq}-H), 2.92-1.89 (1H, m, 2_{eq}-H), 2.65-2.49 (1H, m, 7_{ax}-H), 2.36-2.28 (3H, m, 8_{ax}-H and NCH_2 CH_3), 2.00-1.55 (8H, m, 4_{ax}-, 2_{ax}-, 5-, 8_{eq}-, 7_{eq}-, 6_{ax}-, 6_{eq}- and 9_{ax}-H), 1.25 (3H, t, *J* 7.2, $CO_2CH_2CH_3$) and 1.05 (3H, t, *J* 7.2, NCH_2CH_3); $C_{15}H_{27}NO_3$ requires MW 269. Found: m/z (EI) 269 (M^+, 15%), 224 [$(M-OCH_2CH_3)^+$, 100%].

[See Section 4.2.2.3]

4.3.2.40 3-Ethyl-3-aza-9-(*RS*)-methoxymethyl-bicyclo[3.3.1]nonane-1-methanol (311)

To a stirred solution of methyl ether (310) (mixture of epimers a and b, ~4:3 respectively) (0.80g, 3.0mmol) in anhydrous tetrahydrofuran (30cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added dropwise (15min) a suspension of lithium aluminium hydride in anhydrous diethyl ether (3.0cm^3, 1M, 3.0mmol). The resulting suspension was stirred until completion of the reaction (2h). Ethyl acetate (5cm^3) and then water (2 x 5cm^3) were successively added (exothermal-caution) to the mixture at 25°C, in order to decompose the excess of hydride after which aqueous hydrochloric acid solution (2M, 6cm^3) was added to the resulting white precipitate. The acidic mixture was then basified to pH 9 (saturated aqueous sodium hydrogen carbonate solution 40cm^3) and the resulting mixture was extracted with ethyl acetate (2 x 100cm^3). The combined organic layers were washed with brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual colourless oil was purified over silica gel (80g) eluted with ethyl acetate-hexane (3:1) to give the 9-(*RS*)-methoxymethyl substituted alcohol (311) as a colourless oil (0.61g, 90%) (mixture of epimers at C-9). TLC (ethyl acetate-hexane 3:1, $R_f = 0.3$); υ_{max} (film)/cm^{-1}: 3454s, 2930m, 2852m, 1458w, 1443m and 1120s; δ_H ($CDCl_3$)/ppm (270MHz) 3.56-3.45 (2H, m, CHC*H*$_2$ OCH_3), 3.36 (3H, s, $CHCH_2OC$*H*$_3$), 3.31-2.89 (3H, m, 4$_{eq}$-H and C*H*$_2$ OH), 2.73-2.57 (2H, m, 2$_{eq}$- and 7$_{ax}$-H), 2.35 (2H, q, *J* 7.2, NC*H*$_2$ CH_3), 2.31-2.06 (3H, m, 4$_{ax}$-, 2$_{ax}$- and 8$_{ax}$-H), 1.77-1.41 (7H, m, OH, 5-, 9-, 8$_{eq}$-, 7$_{eq}$-, 6$_{ax}$- and 6$_{eq}$-H) and 1.05 (3H, t, *J* 7.2, NCH_2C*H*$_3$); $C_{13}H_{25}NO_2$ requires MW 227. Found: m/z (EI) 227 (M^+, 1%), 212 [$(M-CH_3)^+$, 100%]. [See Section 4.2.2.3]

4.3.2.41 3-Ethyl-3-aza-9-(*RS*)-methoxymethyl-bicyclo[3.3.1]nonane-1-methyl 2-aminobenzoate (312)

To a stirred solution of isatoic anhydride (248) (0.33g, 2.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.03g, 0.2mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (5cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added substituted primary alcohol (311) (mixture of epimers) (0.50g, 2.2mmol, 1.1equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring was continued until completion of the reaction (22h). The mixture was cooled to 25°C (20min) and then partitioned between ethyl acetate (20cm^3) and water (20cm^3). The aqueous layer was extracted with ethyl acetate (3 x 20cm^3) and the combined organic layers were then washed successively with water (3 x 50cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual brown oil was purified over silica gel (50g) eluted with 3% methanol in dichloromethane to give the 2-aminobenzoate (312) as a colourless oil (450mg, 65%) (mixture of epimers at C-9 a and b, 5:3 respectively). TLC (dichloromethane, R_f = 0.25 and 0.20); υ_{max} (film)/cm^{-1}: 3461s, 2820m, 1684s, 1620w, 1465w, 1452m, 1294w, 1160w and 780w; Fast eluting epimer (312a): δ_H ($CDCl_3$)/ppm (270MHz) 7.82 (1H, d, *J* 8.1, 6'-H), 7.28 (1H, t, *J* 8.1, 4'-H), 6.70-6.62 (2H, m, 3'- and 5'-H), 5.70 (2H, br s, NH_2), 3.98-3.93 (2H, m, $ArCO_2CH_2$ R), 3.60-3.50 (2H, m, CH_2 OCH_{3ax}), 3.36 (3H, s, $CH_2OCH_{3\ ax}$), 3.04-2.96 (2H, m, 2_{eq}- and 4_{eq}-H), 2.74-2.52 (1H, m, 7_{ax}-H), 2.44-2.38 (2H, m, NCH_2 CH_3), 2.15-2.08 (2H, m, 2_{ax}- and 4_{ax}-H), 1.94-1.83 (1H, m, 5-H), 1.79-1.41 (6H, m, 9_{eq}-, 8_{ax}-, 8_{eq}-, 7_{eq}-, 6_{ax}- and 6_{eq}-H) and 1.05-1.02 (3H, m, NCH_2CH_3); Slow eluting epimer (312b): δ_H ($CDCl_3$)/ppm (270MHz) 7.82 (1H, d, *J* 8.1, 6'-H), 7.28 (1H, t, *J* 8.1, 4'-H), 6.70-6.62 (2H, m, 3'- and 5'-H), 5.70 (2H, br s, NH_2), 3.98-3.93 (2H, m, $ArCO_2CH_2$ R), 3.60-3.50 (2H, m, CH_2 OCH_{3eq}), 3.36 (3H, s, $CH_2OCH_{3\ eq}$), 3.16 (1H, d, *J* 6.3, 2_{eq}-H), 3.04-2.96 (1H, m, 4_{eq}-H), 2.74-2.52 (2H, m, 7_{ax}- and 2_{ax}-H), 2.44-2.38 (2H, m, NCH_2 CH_3), 1.94-1.83 (1H, m, 5-H), 1.79-1.41 (7H, m,

4_{ax}-, 9_{ax}-, 8_{ax}-, 8_{eq}-, 7_{eq}-, 6_{ax}- and 6_{eq}-H) and 1.05-1.02 (3H, m, NCH_2CH_3); $C_{20}H_{30}N_2O_3$ requires MW 346. Found: m/z (EI) 346 (M^+, 5%), 331 [$(M-CH_3)^+$, 100%]. [See Sections 4.2.2.1, 4.2.3.1 and 4.2.3.4]

4.3.2.42 3-Ethyl-3-aza-9-(*RS*)-methoxymethyl-bicyclo[3.3.1]nonane-1-methyl [2-(*RS*)-methylsuccinimido]benzoate (313)

A mixture of 2-aminobenzoate (312) (mixture of epimers a and b ~5:3 respectively) (346mg, 1.0mmol) and (*RS*)-methylsuccinic anhydride (257) (228mg, 2.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (6h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (15cm^3) and saturated aqueous sodium hydrogen carbonate solution (15cm^3). The aqueous layer was extracted with ethyl acetate (3 x 25cm^3) and the combined organic layers were washed successively with saturated aqueous sodium hydrogen carbonate solution (2 x 25cm^3) and brine (20cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual golden oil was purified over silica gel (20g) eluted with ethyl acetate-hexane (1:2) to give the [2-(*RS*)-methylsuccinimido]benzoate (313) as a colourless oil (265mg, 60%) (mixture of isomers a and b, 3:2 respectively). TLC (ethyl acetate-hexane 1:3, R_f = 0.35 and 0.30); υ_{max} (film)/cm^{-1}: 2879s, 1782w, 1720s, 1601w, 1490m, 1472m, 1456m, 1390s, 1265s, 1184w, 1096m and 745w; Fast eluting isomer (313a): δ_H ($CDCl_3$)/ppm (270MHz) 8.13 (1H, d, *J* 7.8, 6'-H), 7.65 (1H, t, *J* 7.8, 4'-H), 7.52 (1H, t, *J* 7.8, 5'-H), 7.24 (1H, d, *J* 8.0, 3'-H), 4.35-4.29 (2H, m, $ArCO_2CH_2$ R), 3.61-3.47 (3H, m, 4_{eq}-H and CH_2 OCH_{3ax}), 3.30 (3H, s, $CH_2OCH_{3\ ax}$), 3.12-2.87 (3H, m, 2_{eq}-H and 3"-H_2), 2.75-2.45 (4H, m, 7_{ax}-, 2"-H and NCH_2 CH_3), 2.25-2.05 (2H, m, 2_{ax}- and 4_{ax}-H), 1.95-1.45 (10H, m, 8_{ax}-, 8_{eq}-, 7_{eq}-, 6_{ax}-, 6_{eq}-, 5-, 9_{eq}-H and 5"-H_3) and 1.02 (3H, t, *J* 7.2, NCH_2CH_3); Slow eluting isomer (313b): δ_H ($CDCl_3$)/ppm (270MHz) 8.13 (1H, d, *J* 7.8, 6'-H), 7.65 (1H, t, *J* 7.8, 4'-H), 7.52 (1H, t, *J* 7.8, 5'-H), 7.24 (1H, d, *J* 7.9, 3'-H), 4.35-4.29 (2H, m, $ArCO_2CH_2$ R), 3.61-3.47 (2H, m, CH_2 OCH_{3eq}), 3.30 (3H, s,

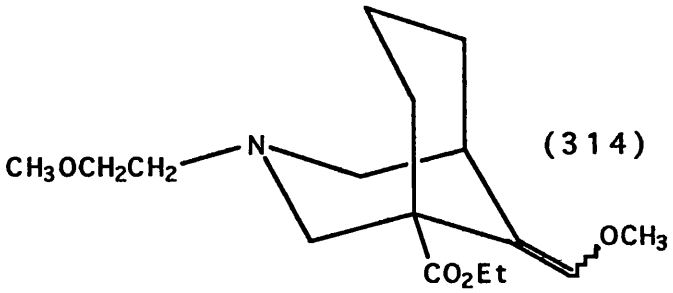
CH_2OCH_3 $_{eq}$), 3.12-2.87 (2H, m, 3"-H_2), 2.75-2.45 (6H, m, 2_{eq}-, 4_{eq}-, 7_{ax}-, 2"-H and NCH_2 CH_3), 2.41-2.22 (2H, m, 2_{ax}- and 4_{ax}-H), 1.95-1.45 (10H, m, 8_{ax}-, 8_{eq}-, 7_{eq}-, 6_{ax}-, 6_{eq}-, 5-, 9_{ax}-H and 5"-H_3) and 1.02 (3H, t, *J* 7.2, NCH_2CH_3); δ_C ($CDCl_3$)/ppm (100MHz) 179.9 (C-1"), 176.2 (C-4"), 164.1 (OC=O), 133.4 (C-4'), 132.8 (C-2'), 131.4 (C-6'), 129.9 (C-3'), 129.4 (C-5'), 127.6 (C-1'), 81.0 (C-9), 70.1 ($ArCO_2CH_2R$), 61.2 (C-2), 58.7 (C-4), 59.2 (CH_2OCH_3), 55.7 (CH_2OCH_3), 52.2 (NCH_2CH_3), 39.1 (C-1), 37.0 (C-3"), 35.4 and 35.2 (C-2", both isomers), 31.6 (C-5), 29.3 (C-8), 29.0 (C-6), 21.0 (C-7), 16.5 and 16.3 (C-5", both isomers) and 12.7 (NCH_2CH_3); $C_{25}H_{34}N_2O_5$ requires MW 442 and C, 67.8; H, 7.7; N, 6.3%. Found: m/z (EI) 442 (M^+, 10%), 397 [$(M-CH_2OCH_3)^+$, 100%] and C, 68.0; H, 7.7; N, 6.2%.

[See Sections 4.2.2.2, 4.2.3.2 and 4.2.3.4]

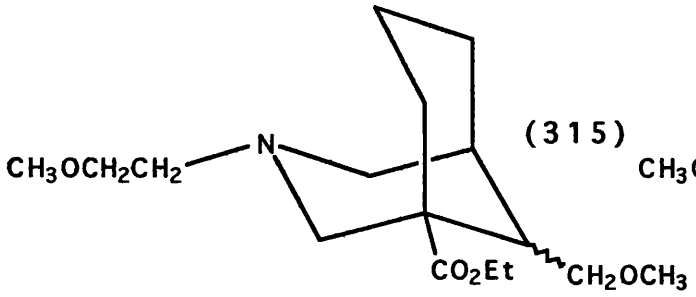
4.3.2.43 Ethyl 3-(2-methoxyethyl)-3-aza-9-(*EZ*)-methoxymethylidene-bicyclo[3.3.1]nonane-1-carboxylate (314)

To a stirred suspension of (methoxymethyl)triphenylphosphonium chloride (308) (12.86g, 37.5mmol, 1.5 equiv.) in anhydrous tetrahydrofuran (75cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added ^{n}butyllithium as a solution in hexane (23.4cm^3, 1.6M, 37.5mmol, 1.5 equiv.). After stirring the resulting deep red suspension (30min), a solution of ketoester (302) (6.73g, 25.0mmol) in anhydrous tetrahydrofuran (38cm^3) was then added with stirring over 20min. The stirring was continued until completion of the reaction (16h) and the reaction mixture was then added to saturated aqueous ammonium chloride solution (100cm^3). The aqueous layer was extracted with ethyl acetate (3 x 75cm^3) and the combined organic layers were washed with brine (75cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual dark red oil was then diluted with diethyl ether (28cm^3) and cooled to 5°C (16h). The mixture was then filtered in order to remove the precipitated triphenylphosphine oxide and the residue was washed with cold (5°C) diethyl ether (125cm^3). The filtrate was concentrated under reduced pressure and the residual dark orange

(308)



(314)



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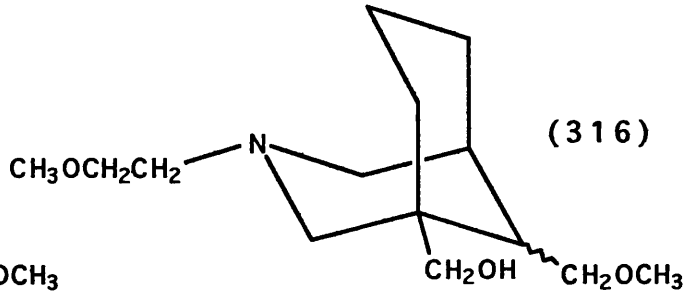

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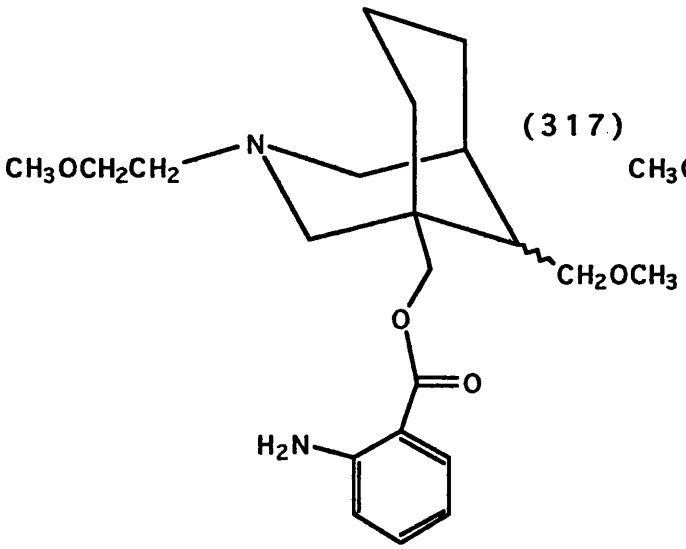
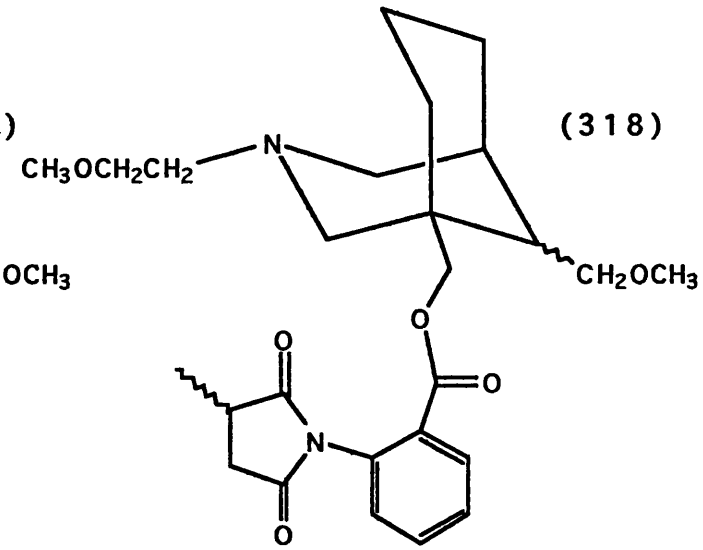


(317)



(318)

oil was purified over silica gel (175g) eluted with diethyl ether-hexane (1:4) to give the (*E/Z*)-enol ether (314) as a pale yellow oil (3.27g, 44%) (mixture of *Z* and *E* isomers, 4:3 respectively). TLC (diethyl ether-hexane 1:1, R_f = 0.3 and 0.2); υ_{max} (film)/cm^{-1}: 3020m, 2820m, 1725s, 1680s, 1240s, 1030s and 800m; Fast eluting isomer (314a): δ_H ($CDCl_3$)/ppm (270MHz) 5.79 (1H, s, C*H*aOCH_3), 4.11 (2H, q, *J* 7.3, CO_2CH_2 CH_3), 3.52-3.48 (2H, m, NCH_2CH_2 OCH_3), 3.44 (3H, s, CHOCH_3 a), 3.35 (3H, s, $NCH_2CH_2OCH_3$), 2.96 (1H, d, *J* 8.0, 4_{eq}-H), 2.94-2.90 (1H, m, 2_{eq}-H), 2.82-2.66 (2H, m, 2_{ax}- and 7_{ax}-H), 2.47 (2H, t, *J* 6.5, NCH_2 CH_2OCH_3), 2.31-2.26 (2H, m, 4_{ax}- and 8_{ax}-H), 2.16-2.05 (1H, m, 5-H), 1.95-1.62 (3H, m, 8_{eq}-, 6_{eq}- and 6_{ax}-H), 1.57-1.46 (1H, m, 7_{eq}-H) and 1.28 (3H, t, *J* 7.3, $CO_2CH_2CH_3$); Slow eluting isomer (314b): δ_H ($CDCl_3$)/ppm (270MHz) 5.68 (1H, s, C*H*bOCH_3), 4.18 (2H, q, *J* 7.3, CO_2CH_2 CH_3), 3.54 (3H, s, CHOCH_3 b), 3.52-3.48 (2H, m, NCH_2CH_2 OCH_3), 3.35 (3H, s, $NCH_2CH_2OCH_3$), 3.05 (1H, d, *J* 10.9, 4_{eq}-H), 3.02-2.99 (2H, m, 7_{ax}- and 5-H), 2.94-2.90 (1H, m, 2_{eq}-H), 2.59 (1H, d, *J* 10.1, 4_{ax}-H), 2.47 (2H, t, *J* 6.5, NCH_2 CH_2OCH_3), 2.31-2.26 (1H, m, 2_{ax}-H), 2.16-2.05 (1H, m, 8_{ax}-H), 1.95-1.62 (3H, m, 8_{eq}-, 6_{eq}- and 6_{ax}-H), 1.57-1.46 (1H, m, 7_{eq}-H) and 1.28 (3H, t, *J* 7.3, $CO_2CH_2CH_3$); $C_{16}H_{27}NO_4$ requires MW 297. Found: m/z (EI) 297 (M^+, 1%), 252 [$(M-OCH_2CH_3)^+$, 100%]. [See Section 4.2.2.3]

4.3.2.44 Ethyl 3-(2-methoxyethyl)-3-aza-9-(*RS*)-methoxymethyl-bicyclo[3.3.1]nonane-1-carboxylate (315)

A solution of enol ether (314) (mixture of *E/Z* geometrical isomers a and b, ~4:3 respectively) (2.97g, 10.0mmol) in anhydrous 1,2-dimethoxyethane (35cm^3) was stirred with palladium on charcoal (10%, 3.50g) under hydrogen (5 atm.) at 25°C until completion of the reaction (2h). The mixture was filtered through celite and the residue was washed with diethyl ether (120cm^3). The filtrate was concentrated under reduced pressure and the residual yellow oil was purified over silica gel (150g) eluted with diethyl ether-hexane (4:1) to give the 9-(*RS*)-

methoxymethyl ether (315) as a light golden oil (1.76g, 59%) (mixture of epimers at C-9 a and b, 3:2 respectively). TLC (diethyl ether-hexane 1:4, R_f = 0.3 and 0.2); υ_{max} (film)/cm^{-1}: 2915s, 1725s, 1460m, 1365m, 1230m, 1120s, 965w, 855w and 735w; Fast eluting epimer (315a): δ_H ($CDCl_3$)/ppm (270MHz) 4.15-4.06 (2H, m, CO_2CH_2 CH_3), 3.52-3.37 (4H, m, NCH_2CH_2 OCH_3 and $CHCH_2$ OCH_{3ax}), 3.34 (3H, s, $CHCH_2OCH_3$ $_{ax}$), 3.30 (3H, s, $NCH_2CH_2OCH_3$), 2.95 (1H, d, 11.9, 2_{eq}-H), 2.90 (1H, d, J 10.6, 4_{eq}-H), 2.66-2.42 (4H, m, 7_{ax}-, 4_{ax}-H and NCH_2 CH_2OCH_3), 2.45 (1H, d, J 6.0, 2_{ax}-H), 2.36-2.32 (1H, m, 8_{ax}-H), 2.00-1.47 (6H, m, 5-, 8_{eq}-, 7_{eq}-, 6_{ax}-, 6_{eq}- and 9_{eq}-H) and 1.25 (3H, t, J 7.1, $CO_2CH_2CH_3$); Slow eluting epimer (315b): δ_H ($CDCl_3$)/ppm (270MHz) 4.15-4.06 (2H, m, CO_2CH_2 CH_3), 3.52-3.37 (4H, m, NCH_2CH_2 OCH_3 and $CHCH_2$ OCH_{3eq}), 3.35 (3H, s, $CHCH_2OCH_3$ $_{eq}$), 3.29 (3H, s, $NCH_2CH_2OCH_3$), 2.90 (1H, d, J 10.6, 4_{eq}-H), 2.66-2.42 (6H, m, 7_{ax}-, 4_{ax}-, 2_{eq}-, 2_{ax}-H and NCH_2 CH_2OCH_3), 2.36-2.32 (1H, m, 8_{ax}-H), 2.00-1.47 (6H, m, 5-, 8_{eq}-, 7_{eq}-, 6_{ax}-, 6_{eq}- and 9_{ax}-H) and 1.25 (3H, t, J 7.1, $CO_2CH_2CH_3$); $C_{16}H_{29}NO_4$ requires MW 299. Found: m/z (EI) 299 (M^+, 5%), 254 [(M-OCH_2CH_3)$^+$, 100%]. [See Section 4.2.2.3]

4.3.2.45 3-(2-Methoxyethyl)-3-aza-9-(*RS*)-methoxymethyl-bicyclo[3.3.1]nonane-1-methanol (316)

To a stirred solution of methyl ether (315) (mixture of epimers a and b, ~3:2 respectively) (0.90g, 3.0mmol) in anhydrous tetrahydrofuran (30cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added dropwise (15min) a suspension of lithium aluminium hydride in anhydrous diethyl ether (3.0cm^3, 1M, 3.0mmol). The resulting suspension was stirred until completion of the reaction (2h). Ethyl acetate (5cm^3) and then water (2 x 5cm^3) were successively added (exothermal-caution) to the mixture at 25°C, in order to decompose the excess of hydride after which aqueous hydrochloric acid solution (2M, 6cm^3) was added to the resulting white precipitate. The acidic mixture was then basified to pH 9 (saturated aqueous sodium hydrogen carbonate solution

40cm^3) and the resulting mixture was extracted with ethyl acetate (2 x 100cm^3). The combined organic layers were washed with brine (100cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual colourless oil was purified over silica gel (80g) eluted with ethyl acetate to give the 9-(*RS*)-methoxymethyl substituted alcohol (316) as a colourless oil (0.77g, 100%) (mixture of epimers at C-9 a and b, 8:5 respectively). TLC (ethyl acetate, R_f = 0.3); υ_{max} (film)/cm^{-1}: 3450s, 2930m, 1460m, 1275s and 1120s; δ_H ($CDCl_3$)/ppm (270MHz) 3.58-3.44 (4H, m, $CHCH_2$ OCH_3 and NCH_2CH_2 OCH_3), 3.37 (3H, s, $CHCH_2OCH_3$), 3.34 (3H, s, $NCH_2CH_2OCH_3$), 3.30-2.90 (4H, m, 4_{eq}-, 2_{eq}-H and CH_2 OH), 2.71-2.57 (2H, m, 7_{ax}- and 4_{ax}-H), 2.42 (2H, br t, *J* 6.0, NCH_2 CH_2OCH_3), 2.30-2.09 (2H, m, 2_{ax}- and 8_{ax}-H) and 1.78-1.39 (7H, m, OH, 5-, 9-, 8_{eq}-, 7_{eq}-, 6_{ax}- and 6_{eq}-H); $C_{14}H_{27}NO_3$ requires MW 257. Found: m/z (EI) 257 (M^+, 5%), 212 [$(M-CH_2OCH_3)^+$, 100%].

[See Section 4.2.2.3]

4.3.2.46 3-(2-Methoxyethyl)-3-aza-9-(*RS*)-methoxymethyl-bicyclo[3.3.1]nonane-1-methyl 2-aminobenzoate (317)

To a stirred solution of isatoic anhydride (248) (0.33g, 2.0mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (249) (0.03g, 0.2mmol, 0.1 equiv.) in anhydrous *N,N*-dimethylformamide (5cm^3) at 25°C, under an atmosphere of anhydrous nitrogen, was added substituted primary alcohol (316) (mixture of epimers a and b, 8:5 respectively) (0.57g, 2.2mmol, 1.1equiv.) in one portion. The solution was then heated to 60°C (oil-bath) and the stirring was continued until completion of the reaction (22h). The mixture was cooled to 25°C (20min) and then partitioned between ethyl acetate (20cm^3) and water (20cm^3). The aqueous layer was extracted with ethyl acetate (3 x 20cm^3) and the combined organic layers were then washed successively with water (3 x 50cm^3) and brine (50cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual brown oil was purified over silica gel (50g) eluted with 3% methanol in dichloromethane to give the 2-aminobenzoate (317) as a colourless oil (639mg,

85%) (mixture of epimers at C-9 a and b, 4:3 respectively). TLC (dichloromethane, R_f = 0.35 and 0.30); υ_{max} (film)/cm^{-1}: 3465s, 2820m, 1680s, 1620m, 1465w, 1295w, 1245s, 1160w, 1103m and 780w; δ_H ($CDCl_3$)/ppm (270MHz) 7.84 (1H, d, *J* 8.0, 6'-H), 7.27 (1H, t, *J* 8.0, 4'-H), 6.69-6.63 (2H, m, 3'- and 5'-H), 5.72 (2H, br s, NH_2), 3.95 (2H, q, *J* 7.1, $ArCO_2CH_2$ R), 3.63-3.59 (2H, m, CH_2 OCH_3), 3.58-3.48 (2H, m, CH_2 OCH_3), 3.35 (3H, s, $CHCH_2OCH_3$), 3.32 (3H, s, $NCH_2CH_2OCH_3$), 3.03-2.93 (2H, m, 4_{eq}- and 2_{eq}-H), 2.67-2.62 (1H, m, 7_{ax}-H), 2.47-2.43 (2H, m, NCH_2 CH_2OCH_3), 2.31-2.21 (2H, m, 2_{ax}- and 4_{ax}-H), 1.96-1.83 (1H, m, 5-H) and 1.78-1.43 (6H, m, 9-, 8_{ax}-, 8_{eq}-, 7_{eq}-, 6_{ax}- and 6_{eq}-H); $C_{21}H_{32}N_2O_4$ requires MW 376. Found: m/z (EI) 376 (M^+, 1%), 331 [$(M-CH_2OCH_3)^+$, 100%].

[See Sections 4.2.2.1, 4.2.3.1 and 4.2.3.4]

4.3.2.47 3-(2-Methoxyethyl)-3-aza-9-(*RS*)-methoxymethyl-bicyclo[3.3.1]nonane-1-methyl [2-(*RS*)-methylsuccinimido]benzoate (318)

A mixture of 2-aminobenzoate (317) (mixture of epimers a and b, ~4:3 respectively) (384mg, 1.0mmol) and (*RS*)-methylsuccinic anhydride (257) (228mg, 2.0mmol, 2.0 equiv.) was stirred at 125°C (oil-bath), under an atmosphere of nitrogen, until completion of the reaction (5h). The resulting dark brown gum was cooled to 25°C (30min) and then partitioned between ethyl acetate (15cm^3) and saturated aqueous sodium hydrogen carbonate solution (15cm^3). The aqueous layer was extracted with ethyl acetate (3 x 25cm^3) and the combined organic layers were washed successively with saturated aqueous sodium hydrogen carbonate solution (2 x 25cm^3) and brine (20cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residual golden oil was purified over silica gel (20g) eluted with ethyl acetate-hexane (1:2) to give the [2-(*RS*)-methylsuccinimido]benzoate (318) as a colourless oil (142mg, 30%) (mixture of isomers). TLC (ethyl acetate-hexane 1:2, R_f = 0.40 and 0.35); υ_{max} (film)/cm^{-1}: 2880s, 1780w, 1720s, 1601w, 1490m, 1455m, 1390s, 1265s,

1185m, 1095m and 745w; δ_H ($CDCl_3$)/ppm (270MHz) 8.12 (1H, d, *J* 8.0, 6'-H), 7.66 (1H, t, *J* 8.0, 4'-H), 7.53 (1H, t, *J* 8.0, 5'-H), 7.25 (1H, d, *J* 8.0, 3'-H), 4.33-4.28 (2H, m, $ArCO_2CH_2$ R), 3.59-3.48 (4H, m, NCH_2CH_2 OCH_3 and $CHCH_2$ OCH_3), 3.30 (3H, s, $CHCH_2OCH_3$), 3.28 (3H, s, $NCH_2CH_2OCH_3$), 3.11-2.88 (4H, m, 2_{eq}-, 4_{eq}-H and 3"-H_2), 2.72-2.45 (4H, m, 7_{ax}-, 2"-H and NCH_2 CH_2OCH_3), 2.40-2.17 (2H, m, 2_{ax}- and 4_{ax}-H) and 1.96-1.43 (10H, m, 8_{ax}-, 8_{eq}-, 7_{eq}-, 6_{ax}-, 6_{eq}-, 5-, 9-H and 5"-H_3); δ_C ($CDCl_3$)/ppm (100MHz) 179.9 (C-1"), 176.1 (C-4"), 164.1 (OC=O), 133.3 (C-4'), 132.9 (C-2'), 131.3 (C-6'), 129.9 (C-3'), 129.3 (C-5'), 127.6 (C-1'), 81.0 (C-9), 70.2 ($ArCO_2CH_2R$), 61.3 (C-2), 60.4 (C-4), 59.2 ($CHCH_2OCH_3$ and $NCH_2CH_2OCH_3$), 58.2 and 55.6 ($NCH_2CH_2OCH_3$ and $CHCH_2OCH_3$), 42.3 ($NCH_2CH_2OCH_3$), 39.1 (C-1), 37.0 (C-3"), 35.4 and 35.2 (C-2", both isomers), 31.6 (C-5), 30.3 (C-8), 29.1 (C-6), 21.1 (C-7) and 16.5 and 16.3 (C-5", both isomers); $C_{26}H_{36}N_2O_6$ requires MW 472 and C, 66.1; H, 7.7; N, 5.9%. Found: m/z (EI) 472 (M^+, 1%), 427 [$(M-CH_2OCH_3)^+$, 100%] and C, 65.9; H, 7.7; N, 6.0%.

[See Sections 4.2.2.2, 4.2.3.2 and 4.2.3.4]

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APPENDICES

Appendix 1

Part of the work described in this thesis has appeared in the following publications:

Philippa A. Coates, Ian S. Blagbrough, David J. Hardick, Michael G. Rowan, Susan Wonnacott, and Barry V. L. Potter, Rapid and Efficient Isolation of the Nicotinic Antagonist Methyllycaconitine from *Delphinium*: Assignment of the Methylsuccinimide Absolute Stereochemistry as *S*, *Tetrahedron Letters*, 1994, *35* (46), 8701-8704.

Ian S. Blagbrough, Philippa A. Coates, David J. Hardick, Terence Lewis, Michael G. Rowan, Susan Wonnacott, and Barry V. L. Potter, Acylation of Lycoctonine: Semi-Synthesis of Methyllycaconitine, Inuline and Delsemine Analogues, *Tetrahedron Letters*, 1994, *35* (46), 8705-8708.

Philippa A. Coates, Ian S. Blagbrough, Michael G. Rowan, Barry V. L. Potter, David P. J. Pearson, and Terence Lewis, Rapid and Efficient Entry to Substituted 2-Succinimidobenzoate-3-azabicyclo[3.3.1]nonanes: AE-Bicyclic Analogues of Methyllycaconitine, *Tetrahedron Letters*, 1994, *35* (46), 8709-8712.

Philippa A. Coates, Ian S. Blagbrough, Terence Lewis, Barry V. L. Potter, and Michael G. Rowan, An HPLC Assay for the Norditerpenoid Alkaloid Methyllycaconitine, a Potent Nicotinic Acetylcholine Receptor Antagonist, *J. Pharm. Biomed. Analysis*, 1995, *13*, 1541-1544.

Philippa A. Coates, Ian S. Blagbrough, Michael G. Rowan, David P. J. Pearson, Terence Lewis, and Barry V. L. Potter, Preliminary Synthetic Studies of Methyllycaconitine, a Potent Nicotinic Acetylcholine Receptor Antagonist: Rapid Syntheses of AE-Bicyclic Analogues, *J. Pharm. Pharmacol.*, 1996, *48*, 210-213.

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Rapid and Efficient Isolation of the Nicotinic Receptor Antagonist Methyllycaconitine from *Delphinium*: Assignment of the Methylsuccinimide Absolute Stereochemistry as *S*

Philippa A. Coates, Ian S. Blagbrough*, David J. Hardick, Michael G. Rowan, Susan Wonnacott[J] and Barry V. L. Potter

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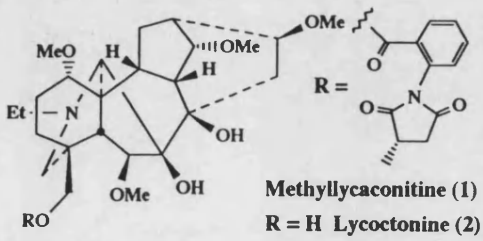
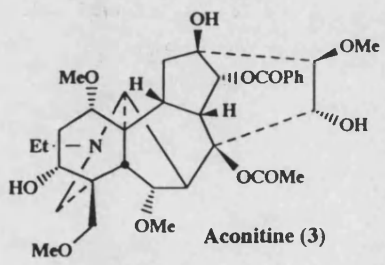
Abstract: Methyllycaconitine (MLA) has been isolated from Garden Hybrid *Delphinium* and purified by vacuum liquid chromatography. ^{13}C NMR and optical rotation have been used to characterize the absolute configuration of the methylsuccinimide moiety as *S*. Ligand binding assays confirmed the potency of MLA and its selectivity for α-bungarotoxin-sensitive neuronal nicotinic acetylcholine receptors.

There is a long history of the use of *Aconitum* and *Delphinium* by various civilizations as sources of poisons and medicines; probably the earliest is the treatment of lice reported by Pliny the Elder.[1] In addition, these plants are held responsible for more cattle deaths in North America than any other poisonous plant.[2] In 1938, Manske examined the aerial portion of *Delphinium brownii* Rydberg and established one of the alkaloids to be methyllycaconitine (MLA) (**1**), the (-)-*N*-(*o*-carboxyphenyl)methylsuccinimide ester of the norditerpenoid alcohol lycoctonine (**2**).[3] MLA (**1**) has been reported in at least 30 *Delphinium* species and also in *Consolida ambigua* and *Inula royaleana*.[4] MLA (**1**) is known to be the principal toxic alkaloid, in these species, and to produce mortality in a broad spectrum of insects.[5] Both its insecticidal action and its toxicity are believed to be a result of nicotinic acetylcholine receptor (nAChR) antagonism and (**1**), at one subset of nAChR, is the most potent, small molecule, competitive antagonist yet reported.[6] Despite one of its trivial names, MLA (**1**) differs from aconitine (**3**) in many respects, and (**1**) has not been found in *Aconitum*.



Methyllycaconitine (1)
R = H Lycoctonine (2)



Aconitine (3)

Due to the high toxicity of norditerpenoid alkaloids[7], the recent use of (**1**) in Russian medicine for its "curariform" activity[8], and the possibilities afforded by (**1**) as a lead compound for the design of pest controlling agents, we require a convenient source of homogeneous (**1**) for structure-activity relationship (SAR) studies. We have therefore undertaken the characterization of (**1**) from a garden hybrid strain of *Delphinium*, closely related to the American cultivar, Pacific Giant, and to the species, *D. elatum*. In this *Letter*, we report a rapid and efficient isolation of the nAChR antagonist (**1**), its saponification to the parent alcohol lycoctonine (**2**), and the unequivocal characterization of the *S*-methylsuccinimide moiety by ^{13}C NMR spectroscopy and by optical rotation.

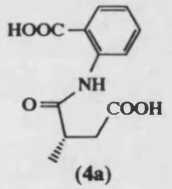
As Benn and his colleagues have consistently drawn[9,10,11], (**1**) apparently contains *S*-(-)-methylsuccinic acid; however, many authors have left the stereochemistry of the substituent on the succinimide as ambiguous. Indeed, as recently as 1989 and 1993, the chirality at this carbon centre has been left undefined and must therefore be supposed to be undefined or insecure.[12,13,14] Early work by Goodson[15] has shown that (-)-methylsuccinic acid is one of the hydrolysis products from (**1**), although the specific optical rotation found was small and no inference was made as to the stereochemistry of the carbon bearing this methyl group. Therefore, we undertook a proof of the configuration of this remaining chiral centre in natural MLA (**1**) to aid in our modelling of the nicotinic pharmacophore for more accurate interpretation of SAR data.

Extraction of Garden Hybrid* Delphinium *seeds and the isolation of MLA (**1**): Seeds of Garden Hybrid *Delphinium* (12 g) were ground and defatted with redistilled hexane (210 ml) in a soxhlet extractor (presoak of 21 h). The seeds were then extracted with redistilled chloroform (180 ml) (presoak of 21 h). Reducing the density of the seeds packed into the soxhlet thimble was found to improve the efficiency of the extraction. The extract was concentrated *in vacuo* (to 50 ml) and then extracted with aqueous sulfuric acid solution (0.75 M, 65 ml). The acidic layer was extracted with redistilled chloroform (2 x 50 ml), basified to pH 10 with saturated aqueous sodium carbonate solution and then extracted with diethyl ether (3 x 50 ml). Drying (Na_2SO_4) and evaporation *in vacuo* of the combined organic layers gave crude alkaloidal material as an off-white foam (147 mg, 1.22% weight of seeds taken); tlc on silica gel (5:4:1 cyclohexane-chloroform-diethylamine, detection by Dragendorff Munier spray) showed 3 main bands. The success of this pilot run was followed by a large scale seed extraction (600 g) with essentially equal efficiency.

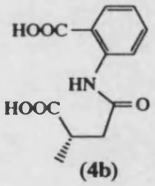
Purification of MLA by vacuum liquid chromatography: Crude alkaloidal material (992 mg), from a large scale seed extraction, was purified by vacuum liquid chromatography[16] over alumina. Elution was performed using a stepped gradient of mixtures of hexane, diethyl ether and methanol, in order of increasing polarity. Fractions were monitored by tlc on silica gel 60. Those fractions containing (**1**) as the sole alkaloid [R_f = 0.30, authentic MLA (**1**) R_f = 0.31, 5:4:1 cyclohexane-chloroform-diethylamine] were combined and evaporated *in vacuo* to yield pure MLA (**1**) (439 mg) homogeneous by tlc and NMR spectroscopy.

Determination of chirality of the methylsuccinimide moiety. Ester (**1**) was saponified with aqueous sodium hydroxide solution to afford lycoctonine (**2**) and the *N*-(methylsuccinyl)anthranilic acids, the half-acid amides (**4a**) and (**4b**), as a mixture of isomers. The diacid (**5**) was then obtained by acid catalysed hydrolysis of (**4a**) and (**4b**) with a little detectable racemization (*vide infra*). The bis-*l*-menthol ester (**6**) of natural (**5**) was prepared[17] and ^{13}C NMR spectroscopic analysis showed that the natural product was the *S*-enantiomer[18] as follows. Racemic-(**5**) was converted into its bis-*l*-menthol ester (**6**) and the methylene carbon of (**5**), i.e. α to the chiral carbon in the succinimide moiety, displayed two signals which could clearly be resolved at 67.8 MHz: R = 37.82 and S = 37.88 ppm (Δ = 0.06 ppm). The chiral carbon signal itself was not resolved into two signals (δ = 36.07 ppm). Diacid (**5**), obtained from (**1**), was converted into (**6**) and displayed peaks at 37.92 and 37.85 ppm (intensity ratio approx. 14:1 respectively).

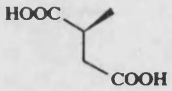
Itaconic acid was hydrogenated in the presence of a $RhCl_3$-BPPM chiral catalyst[19] to afford *S*-(**5**) which was converted into (**6**) whose ^{13}C NMR spectrum displayed 37.94 ppm for the key methylene carbon chemical shift. Inspection of this spectrum, after dilution with one molar equivalent of the bis-*l*-menthol ester (**6**) of racemic (**5**), revealed an additional signal at 37.90 ppm with approximately half the intensity of the higher frequency signal (37.98 ppm; Δ = 0.08 ppm). Additional confirmation of the *S*-configuration at this centre in MLA (**1**) came from the optical rotation of (**5**) obtained from (**1**) *via* (**4a**) and (**4b**). This rotation is small in water ($[\alpha]_D$ = -8.8°, c = 2)[15], but a little larger in ethanol ($[\alpha]_D$ = -15.0°, c = 1.89).[20] Synthetic *S*-(**5**) displayed $[\alpha]_D$ = -14.7° (c = 3.2, EtOH, 25°C). This ^{13}C NMR spectroscopic procedure will be applicable to the analysis of other methylsuccinimides or anhydrides, including half-ester amides and bis-amides.



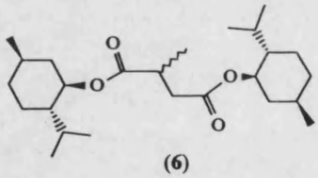
(**4a**)



(**4b**)



(**5**)



(**6**)

Biological activity of purified MLA: The purified MLA (**1**) was assessed for potency at nAChR in ligand binding assays on rat brain membranes.[21] The nAChR subtype identified by [^{125}I]-α-bungarotoxin labelling showed high affinity for (**1**) which inhibited [^{125}I]-α-bungarotoxin binding with a K_i of 3 nM. In contrast, [^{3}H]-nicotine-labelled nAChR bound (**1**) with a K_i of 13 μM. These values agree closely with those previously determined for the citrate salt of (**1**)[22] and confirm the exquisite selectivity of (**1**) for neuronal α-bungarotoxin-sensitive nAChR. The parent alcohol (**2**) exhibited little neuronal blocking action (K_i = 5 μM at [^{125}I]-α-bungarotoxin-labelled nAChR), indicating that MLA's aromatic ester function is a significant haptophore. At [^{3}H]-nicotine labelled nAChR, (**2**) failed to inhibit binding at concentrations up to 1 mM. Therefore, we are investigating the SAR of these alkaloids and, in particular, we are determining the importance of the unusual acyl moiety with respect to potency and selectivity for nAChR subtypes.[23] MLA (**1**) and its many synthetic analogues are useful pharmacological tools as probes for interactions at nAChR.

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Acylation of Lycoctonine: Semi-Synthesis of Inuline, Delsemine Analogues and Methyllycaconitine

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Abstract: Lycoctonine has been acylated to afford sequentially inuline, delsemine analogues and methyllycaconitine using isatoic anhydride followed by *S*-(-)-methylsuccinic anhydride. This protocol is a rapid, facile method for the regiospecific introduction of the anthranilate ester moiety found in potent nicotinic acetylcholine receptor antagonists.

Lycoctonine (**1**) is an important hexacyclic norditerpenoid alkaloid which contains a primary alcohol at C-18 and tertiary alcohols at C-7 and C-8.[1] Modified anthranilate esters of (**1**) at C-18 are highly potent pharmacological agents; in particular, MLA (**2**) is the principal toxic alkaloid of several species of *Delphinium* and produces mortality in cattle[2] as well as in a broad spectrum of insects.[3] Both its insecticidal action and its toxicity are believed to be a result of nicotinic acetylcholine receptor (nAChR) antagonism and, at one subset of nAChR, (**2**) is the most potent, small molecule, competitive nAChR antagonist yet reported.[4] Several research groups are actively studying the structure-activity relationships (SAR) of these alkaloids as probes for selective nAChR subtypes. Esters of (**1**) have been prepared[5], and *N*-deacetyllappaconitine has been acylated on the aniline nitrogen atom with a variety of aliphatic and aromatic carboxylic acids[6] in order to increase the lipophilicity of these potential insecticides.[3] There is no literature precedent for the facile introduction of the natural *S*-anthranoylmethylsuccinimide moiety. Acylation of (**1**) will allow access to the esters inuline (**3**), benzoyllycoctonine (**4**), delsemine (**5a** and **5b**)[7], and the half-ester amides (**6a** and **6b**). The latter may be natural products *per se* or might be artefacts of alcoholysis of (**2**) on isolation.[8]

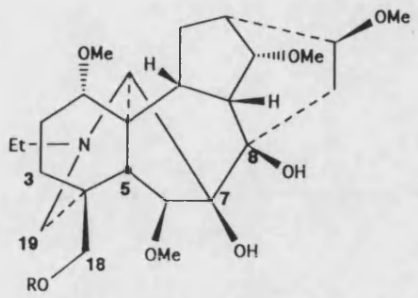
The synthesis of (**3**) can be envisaged by acylation of (**1**)[9], and (**2**) can be derived from (**3**) by formation of the substituted succinimide. In this *Letter*, we report a rapid, facile semi-synthesis of MLA (**2**), via inuline (**3**) and delsemine analogues (**7a** and **7b**) by regiospecific acylation of (**1**). These protocols for the introduction of the anthranilate ester and the subsequent addition of the homochiral succinimide moiety are applicable to the synthesis of related norditerpenoids esterified at C-18 e.g. anhweidelphinine and nudicauline.

The primary alcohol of (**1**) at C-18 should acylate preferentially over the tertiary alcohols at C-7 and C-8. However, it is important to recognize that this nucleophilic alcohol is associated with a neopentyl-like motif and this bulky substituent will affect esterification. We therefore undertook preliminary experiments with neopentyl alcohol (**8**) in order to determine the conditions for the introduction of the anthranilate ester on a milligram scale, suitable for the manipulation of precious natural products. We anticipated that there could be problems attempting to acylate with anthranilic acid, and also with bulky *N*-protecting groups typically used in amino acid chemistry (e.g. tBOC or CBZ) in the *ortho* position of the phenyl ring. These problems can be circumvented by the use of isatoic anhydride which serves to introduce the anthranilate ester with the loss of one equivalent of carbon dioxide. Thus, treatment of (**8**) with (**9**), catalysed by 4-dimethylamino-pyridine (DMAP), in DMF, afforded the anthranilate (**10**) with no trace of the isatoate (**11**) (90°C, 5 h, 64%).[10,11] The C-4-(C-3, C-5, C-19)-C-18 moiety of (**1**) has been mimicked by (**8**) in this successful esterification. Secondary alcohols are sluggish to react with (**9**) and tertiary alcohols are unreactive.[10,11]

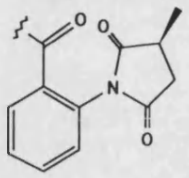
The methylsuccinimide moiety was then introduced by fusion of (**10**) with an excess of neat racemic methylsuccinic anhydride (120°C, 24 h, 79%). Inspection of the ^{1}H NMR spectrum of (**12**) revealed a broad signal for the methyl group (1.46 ppm, half peak height width = 18 Hz), and therefore rotation about the aromatic carbon-nitrogen bond is slow. Further support for this interpretation comes from an inspection of CPK models where the carbonyls on the heterocycle are not free to rotate past the aromatic ester carbonyl functional group. Maleimide (**13**) was prepared (25%) by the fusion of (**10**) with maleic anhydride. This ring closure reaction proceeds via the half-acid amide. Indeed, when succinic anhydride was reacted with (**10**) only (**14**) and not the ring closed product could be isolated. The slow ring-closure step (dehydration) can be accelerated by the addition of carbonyl diimidazole (CDI) (1.2 equiv). The formation of (**12**) and succinimide (**15**) can now be performed from the corresponding anhydride in the presence of CDI, 23 h at 25°C in dichloromethane. In these experiments, racemic methylsuccinic anhydride was used, but, in the synthesis of MLA (**2**), the *S*-enantiomer of methylsuccinic anhydride has been incorporated.[9]

S-(-)-Methylsuccinic acid (**17**) was prepared by hydrogenation of itaconic acid (**16**) in the presence of a $RhCl_3$-BPPM-*S*-methylbenzylamine catalyst[12] (72% yield, >90% ee based on *R*-enantiomer $[\alpha]_D$ = +15.5°).[12] Diacid (**17**) was then converted into anhydride (**18**), $[\alpha]_D$ = -36.5° (c = 3.5, dioxan), with acetyl chloride at 25°C.[13] Lycoctonine (**1**) was esterified with (**9**) catalysed by DMAP, in DMF, 27 h at 70°C, which gave inuline (**3**) (21%). This is the first semi-synthesis of this natural product.[14]

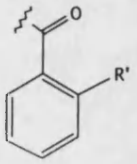
The half-acid amides (**7a**) and (**7b**) were synthesized, but not isolated, by treatment of (**3**) with (**18**) in dichloromethane (28 h at 25°C). The regioisomers (**7a**) and (**7b**) could not be separated by tlc on silica gel 60 using 3:7 methanol-dichloromethane (R_f = 0.8). Closure of the succinimide was achieved by reacting the mixture (**7a**) and (**7b**) with CDI, in dichloromethane, 48 h at 25°C, which gave (**2**), 55% from (**3**), identical in all chromatographic and spectroscopic respects with the natural product.[9,15,16] Thus, the semi-synthesis of MLA (**2**), *via* inuline (**3**), has been achieved by regiospecific acylation of lycoctonine (**1**).



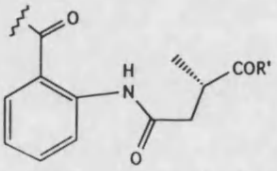
R = H Lycoctonine (1)
(Delsine, Royaline)



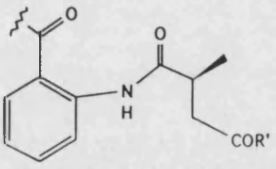
Methyllycaconitine (MLA) (2)
(Delartine, Delsemidine, Mellictine)



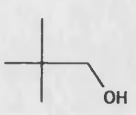
R' = NH_2 Inuline (3)
(Anthranoyllycoctonine)
R' = H Benzoyllycoctonine (4)



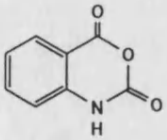
R' = NH_2 Delsemine (5a)
R' = OMe/OEt (6a)
R' = OH (7a)



R' = NH_2 Delsemine (5b)
R' = OMe/OEt (6b)
R' = OH (7b)



(8)



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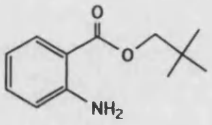

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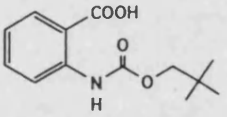

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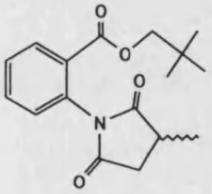

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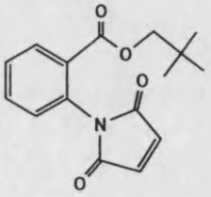

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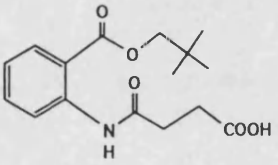

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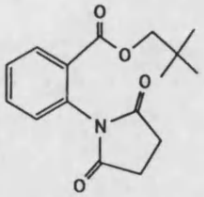

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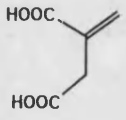

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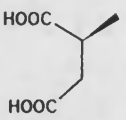

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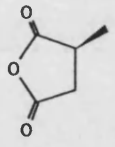

(18)

In ligand binding assays for rat brain nAChR, (**2**) has markedly higher affinity for neuronal α-bungarotoxin binding sites (K_i = 3 nM), i.e. the α7 subtype, than for any other nAChR subtype.[4] We are therefore investigating the SAR of these alkaloids and we are determining the importance of the unusual acyl moiety with respect to selectivity in binding amongst nAChR subtypes. Lycoctonine (**1**) displays low activity at housefly head nAChR (K_i = 380 nM)[3,17] and synthetic delsemine, as a mixture (**5a**) and (**5b**) from the aminolysis of (**2**), is essentially equipotent with (**2**) at a frog extensor-muscle preparation.[18] MLA (**2**) and its synthetic analogues[19,20] are therefore useful pharmacological tools.

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Rapid and Efficient Entry to Substituted 2-Succinimidobenzoate-3-azabicyclo[3.3.1]nonanes: AE-Bicyclic Analogues of Methyllycaconitine

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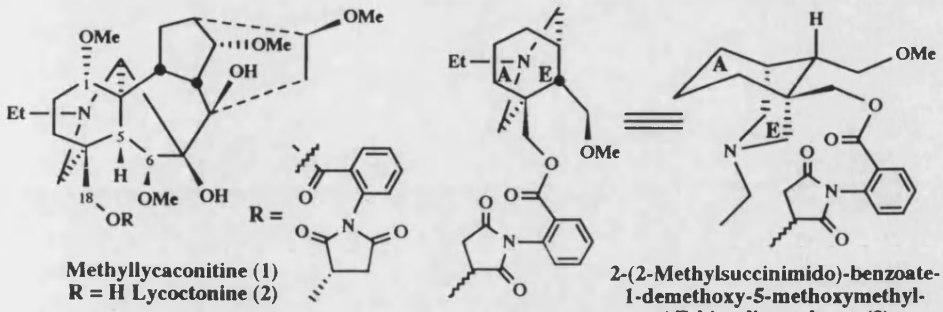
Abstract: A double Mannich reaction allows a rapid and efficient entry to substituted 3-azabicyclo[3.3.1]nonane analogues of methyllycaconitine (MLA), a selective antagonist of certain mammalian and insect α-bungarotoxin-sensitive nicotinic acetylcholine receptors.

Methyllycaconitine (MLA) (**1**) (also known as delartine, delsemidine, and mellictine) is the 2-[2-(*S*)-methylsuccinimido]-benzoate ester of the hexacyclic norditerpenoid alkaloid lycoctonine (**2**) [1] and occurs in *Delphinium* and not *Aconitum*, despite its trivial name. These structurally complex alkaloids contain piperidine (E) and cyclohexane (A) rings in a 3-azabicyclo[3.3.1]nonane motif. Neopentyl-like alcohol (**2**) displays considerably less biological activity than its *N*-substituted anthranilate ester (**1**) [2] which continues to find use in Russian medicine [3] as a muscle relaxant for surgery, as it has curare-like activity and apparently blocks neurotransmission. It has been shown that MLA (**1**) is a potent and selective ligand for neuronal over neuromuscular nicotinic acetylcholine receptors (nAChR).[2] MLA is more selective than the snake toxin α-bungarotoxin used for competitive antagonism of neuronal and neuromuscular nAChR.[2] Furthermore, there is continuing interest in MLA (**1**) and its synthetic analogues, as leads for the design and development of insecticides, acting at insect nAChR.[4] We are therefore undertaking structure-activity relationship (SAR) studies of MLA [5] and, in this *Letter*, we present rapid and efficient syntheses of AE-bicyclic analogues, including the 1-demethoxy-5-methoxymethyl analogue (**3**) (racemic) which contains the piperidine (E) and cyclohexane (A) rings of MLA (**1**) as well as the anthranilate moiety.



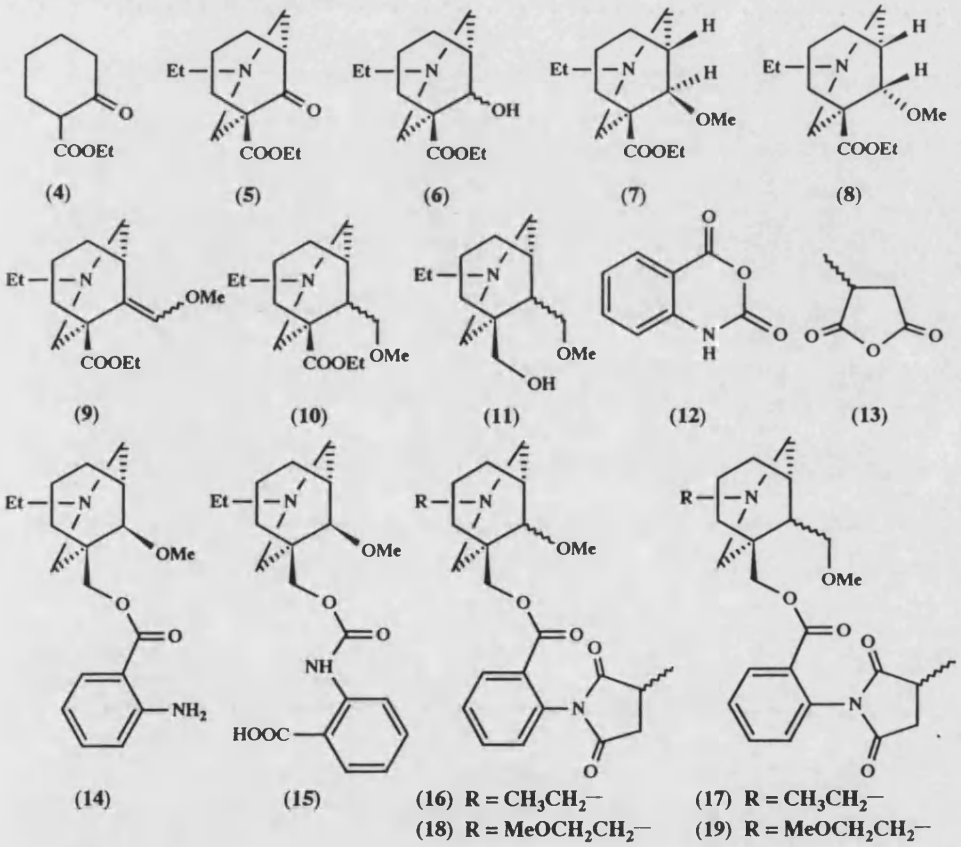
Methyllycaconitine (1)
R = H Lycoctonine (2)

2-(2-Methylsuccinimido)-benzoate-1-demethoxy-5-methoxymethyl-AE-bicyclic analogue (3)

We rationalized that the striking difference in biological activity, more than 10,000 fold, between MLA (**1**) and lycoctonine (**2**) must be, in part, related to the presence of the *N*-succinyl anthranilate ester moiety. In early molecular modelling studies,[6] this ester functional group was compared to the ester found in acetylcholine. The competitive antagonism of nAChR displayed by MLA (**1**) was proposed to be due to the slight distortion of the choline of acetylcholine into the homocholine-like (3-tertiaryaminopropan-1-ol) motif found around the piperidine ring of MLA.[6] Therefore, we have focused our initial synthetic efforts upon the preparation of this functionality by an efficient route which will allow ready access to analogues for biological evaluation. The AE-bicycle is a 3-azabicyclo[3.3.1]nonane which is also found in atisine,[7] cardiopetaline,[8] and related *Aconitum* norditerpenoid alkaloids. Kraus and co-workers have recently published [9] their synthetic approach to the AEBD-tetracycle, and some long-chain fatty acid esters of lycoctonine (**2**) have been prepared by Pelletier and Ross [10] as more lipophilic analogues of MLA (**1**). Benn and Jacyno first reported studies of a monocyclic *N*-methylated piperidine anthranilate ester.[11]

It was immediately apparent that the 3-azabicyclo[3.3.1]nonan-9-one ring system can be prepared from β-keto ester (**4**) which has the correct functionality, appropriately substituted, for the necessary subsequent manipulations. Thus, a double Mannich reaction between keto-ester (**4**), aqueous ethylamine, and aqueous formaldehyde (37%, 2 equiv., EtOH, reflux, 2 h) gave racemic (**5**) (47%).[12] The carboxylic ester carbonyl carbon of (**4**) becomes the neopentyl-type alcohol, equivalent to C-18 of lycoctonine (**2**), of the [3.3.1]bicycle, on reduction and is therefore intrinsic to the design of these MLA (**1**) analogues, not merely a β-keto ester activating group which might later have been removed by decarboxylation. In one series of AE-bicyclic analogues, we decided to exclude oxygenation from C-1 of MLA, and to replace carbon C-6 (norditerpenoid numbering) with an *O*-methyl ether at C-5. Thus, reduction of (**5**) with $NaBH_4$ (EtOH, 25°C, 2 h) gave a mixture of epimeric alcohols (**6**) (81%). *O*-Methylation of (**6**) (NaH, MeI, 1.0 equiv., anhydrous DMF, 25°C, 5 h) gave the required methyl ethers (**7**) (35%) and (**8**) (25%) which were separable on silica gel (hexane-diethyl ether, 2:1) with (**7**) less polar than (**8**), and with no detectable *N*-quaternisation. Characterization of novel ethers (**7**) and (**8**) followed from detailed inspections of their ^{1}H-NMR spectra, in particular, from COSY and nOe experiments as δ 3.53 (d, $J_{5\text{-}9}$ = 3 Hz) and 3.31 (OMe, ax. to cyclohexane) (**7**) and 3.49 (d, $J_{5\text{-}9}$ = 2 Hz) and 3.31 (OMe, eq. to cyclohexane) (**8**) were not diagnostic.

The [3.3.1]bicyclic carbon skeleton can be extended from the ketone at C-5 to C-6 (norditerpenoid numbering) by Grignard- or Wittig-type reactions. In another series of analogues, Wittig reaction of bicyclic cyclohexanone (**5**) was accomplished [nBuLi, (methoxymethyl)triphenylphosphonium chloride, anhydrous THF, 25°C, 16 h] to afford *E*- and *Z-exo*-methylidene *O*-methyl ethers (**9**) (44%). Reduction of this mixture of *E*- and *Z*-enol ethers (**9**) by catalytic hydrogenation (5 atm, 10% Pd/C, anhydrous DME, 25°C, 2 h) gave C-5-methyl-*O*-methyl ether (**10**) as a colourless oil (59%). Conversion of the ethyl ester functional groups, in (**7**), (**8**), and (**10**), into the corresponding neopentyl-type alcohols was smoothly accomplished using LAH (anhydrous THF, 25°C, 2 h) e.g. (**10**) gave (**11**) as a colourless oil (90%). We have developed rapid, practical protocols for the conversion of neopentyl-type alcohols into the corresponding anthranilate esters followed by the efficient introduction of the 2-methylsuccinimide ring, in chiral form,[5] as this moiety of MLA (**1**) is also found in related natural product alkaloids, including *inter alia*: anhweidelphinine, ajacusine, barbinine, elatine, glaudelsine, and nudicauline. Isatoic anhydride (**12**), used under basic conditions, efficiently introduces the anthranilate ester, the presence of a basic catalyst promoting the formation of the ester over the carbamate.[13]



Thus, LAH reduction of ester (**7**) gave the desired alcohol which was reacted with isatoic anhydride (**12**), in the presence of the base DMAP, to give anthranilate (**14**) (anhydrous DMF, 60°C, 16 h, 65%) with no detectable isatoate (**15**). Anthranilates derived from (**7**) and (**8**) were converted into diastereoisomeric 2-methylsuccinimides (**16**) by fusing with anhydride (**13**) (125°C, 3 h, 69%); similarly, Wittig reaction product (**9**) was converted, *via* (**10**) and (**11**), into succinimides (**17**) (60%).[5] The basicity of a tertiary amine can be significantly modulated, lowered by essentially a full pKa unit,[14] by the addition of a β-methoxyethyl group. Therefore, β-methoxyethylamine was incorporated in a series of analogues, potentially more lipophilic at physiological pH, parallel to the *N*-ethyl tertiary amines. The colourless oils (**18**) and (**19**) were efficiently prepared by the above strategy. These AE-bicyclic analogues of MLA (**1**) are being tested at sub-types of mammalian and insect nAChR as part of our continuing SAR studies.

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An HPLC assay for the norditerpenoid alkaloid methyllycaconitine, a potent nicotinic acetylcholine receptor antagonist

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Abstract

The extremely potent and selective nicotinic acetylcholine receptor antagonist methyllycaconitine, MLA, and related norditerpene alkaloids are finding increasing use as neurochemical probes and as targets for structure-activity relationship studies. In this work, an assay procedure for MLA which utilises ion suppression reverse-phase HPLC with UV absorbance detection at 270 nm is described. The method detected 280 ng MLA on column.

Keywords: Reverse-phase chromatography; HPLC; Methyllycaconitine; Ion suppression; Nicotinic acetylcholine receptor antagonist

1. Introduction

Methyllycaconitine (MLA) (Fig. 1) is a norditerpenoid ester alkaloid found in many species of the plant genus *Delphinium* (Ranunculaceae) which is highly toxic both to insects and mammals [1–3]. This toxicity arises from its highly potent and selective antagonist activity at nicotinic acetylcholine receptors [4,5]. We are currently engaged in structure-activity relationship (SAR) studies of MLA and its analogues using other natural alkaloids and semisynthetic derivatives prepared from MLA itself [6–8]. However, the extremely high activity of MLA (inhibitor dissociation constant, $K_i = 2$ nM in a competition binding assay against α-bungarotoxin [4]) means that even low levels of contamination of other test alkaloids with this compound will lead to a significant distortion of bioassay results. Accordingly, we have developed a sensitive HPLC assay for MLA. This practical assay is used to monitor alkaloids and their derivatives prior to biological testing. As MLA and related alkaloids are increasingly finding a use as selective probes in neurochemical studies [9,10], it is important that standard methodologies are developed for the assessment of the chemical purity of these alkaloids.

In addition, the method has been designed to be readily adaptable to LC/MS applications and for scale-up for semi-preparative use. Majak et al. [11] have reported a reverse-phase, ion-pair HPLC system for MLA. However, the external standard method of quantitation they used is insufficiently precise for our purposes, whilst their use of a hexanesulphonate counterion precludes LC/MS

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and scale-up for semi-preparative use. A normal-phase system more recently reported by Manners and Pfister [12] avoids these problems, but their system does not fully resolve MLA from its parent norditerpenoid alcohol, lycoctonine.

Several HPLC methods for the (structurally) closely related *Aconitum* alkaloids have been reported including a reverse-phase ion-pair quantitative assay [13], a qualitative reverse-phase system [14], preparative HPLC on a normal-phase system [15] and LC/MS [16]. In this last report, several of the test alkaloids were poorly resolved chromatographically and identification was achieved by selected-ion monitoring [16].

2. Experimental

2.1. Materials

Seeds of garden hybrid *Delphinium*, a cultivar closely related to the American 'Pacific Giant' and derived predominantly from the species *D. elatum*, were kindly donated by Blackmore and Langdon Ltd., Pensford, Bristol.

An authentic sample of MLA citrate was supplied by Professor M. H. Benn, University of Calgary, Canada. Lappaconitine was purchased from Latoxan, Rosans, France. Reagent grade ammonium acetate and formic acid, and HPLC grade acetonitrile were used in the preparation of the mobile phase. All solvents used in the extraction and separation of the alkaloids were HPLC grade.

2.2. Extraction of alkaloids

Ground, defatted seeds of garden hybrid *Delphinium* were continuously extracted with chloroform in a soxhlet extractor. The extract was concentrated under reduced pressure and partitioned with aqueous sulphuric acid solution (0.1 M). The acid phase was washed with one aliquot of chloroform, then basified to pH 10 with saturated aqueous sodium carbonate solution, and extracted with diethyl ether. After washing with water and drying over anhydrous Na_2SO_4, the ether was removed in vacuo at 40 °C to leave a crude total alkaloid mixture as an off-white foam.

The alkaloid mixture was separated by vacuum liquid chromatography over basic alumina (TLC grade) eluted with a step gradient of hexane, diethyl ether and methanol mixtures of increasing polarity. Delpheline (Fig. 1) was the first major alkaloid eluted followed by MLA and then a mixture of more polar components. Delpheline was further purified by recrystallisation from ethanol:hexane (1:1, v/v).

Lycoctonine (Fig. 1) was prepared by hydrolysis of MLA with 0.1 M NaOH in ethanol for 16 h at 20 °C and purified by recrystallisation from ethanol. The alkaloids were authenticated by comparison of their ^1H NMR, ^{13}C NMR and mass spectra with literature values [1,17].

2.3. Apparatus

HPLC was carried out using a JASCO PU980 pump, a 100 µl Rheodyne injection loop and a JASCO UV975 variable UV detector at 270 nm. A 25 cm × 4.6 mm i.d. Hypersil ODS 5 µm column was used with a mobile phase of 0.2 M aqueous ammonium acetate adjusted to the required pH (3.0–5.0) with formic acid. Acetonitrile was used as organic modifier. All mobile phase mixtures were pumped at a flow rate of 1 ml min^{-1}.

Lappaconitine (Fig. 1), a commercially available norditerpenoid alkaloid with an aromatic chromophore, was used as an internal standard.

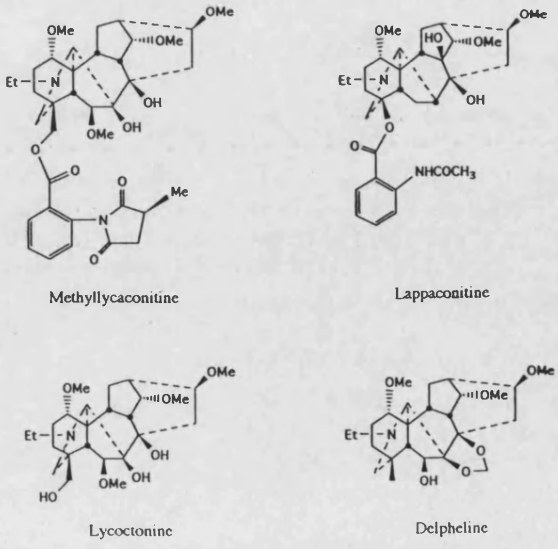

Fig. 1. Chemical structures of MLA, lycoctonine, delpheline and lappaconitine.

2.4. Calibration curves

A calibration curve was constructed by injecting, in duplicate, a series of mixtures consisting of 2.8–100 μg ml^{-1} of MLA with 25 μg ml^{-1} of lappaconitine in mobile phase. MLA peak height over lappaconitine peak height was plotted against MLA concentration and a line fitted by linear regression. Six replicates of solutions containing 5 μg ml^{-1} and 25 μg ml^{-1} of MLA were injected in order to assess the intra-day precision of the method.

2.5. Assays

For the assay of MLA in a crude alkaloid extract from cultivated *Delphinium* seed, approximately 2 mg of the extract, accurately weighed, was dissolved in mobile phase (10 ml). Aliquots (1 ml) were taken and mixed with 1 ml of a 0.05 mg ml^{-1} solution of lappaconitine in mobile phase. For the assay of MLA in purified alkaloid samples, approximately 10 mg of the alkaloid, accurately weighed, was dissolved in mobile phase (1 ml), and mixed with 1 ml of a 0.05 mg ml^{-1} lappaconitine solution.

3. Results and discussion

3.1. Optimisation of mobile phase

The aim was to develop a reverse-phase system which utilised a volatile mobile phase which would provide the flexibility for routine analytical use and also be adaptable for LC/MS and for semi-preparative HPLC. The use of a large organic anionic counterion, as employed in previous chromatographic methods for norditerpenoid alkaloids [11,13], was precluded by this requirement, but ionisation of these relatively weak bases was suppressed by buffering the mobile phase at relatively high pH values. Increasing the pH from 3.0 to 4.0 to 5.0 increased the capacity ratio, K', for MLA from 2.0 to 3.8 to 6.0 respectively when the acetonitrile content of the mobile phase was maintained at 35% (v/v). However, storage of samples in mobile phase at pH 3 showed progressive loss of MLA with more than 50% lost after 3 months and the concomitant appearance of several extra peaks in the chromatogram. At pH 5, MLA showed slight instability on prolonged storage, but no loss and no breakdown products were detected during the course of an intra-day precision experiment. Accordingly,

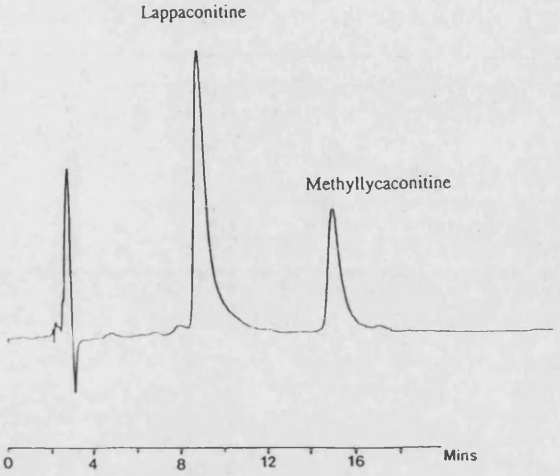

Fig. 2. A chromatogram obtained for 25 μg ml^{-1} of MLA and 25 μg ml^{-1} of lappaconitine using a 25 cm × 4.6 mm column of Hypersil ODS 5 μm and a mobile phase of 0.2 M ammonium acetate adjusted to pH 5 with formic acid:acetonitrile (70:30, v/v), 1 ml min^{-1} and detection by UV absorbance at 270 nm.

pH 5 was chosen for routine use, care being taken to prepare the injection solutions immediately prior to chromatography. Increasing the acetonitrile content from 25% (v/v) to 30% (v/v) to 35% (v/v) to 40% (v/v) at a constant pH of 4.0 led to a decrease in the K' value from 9.5 to 4.4 to 2.9 to 2.5 respectively. A mobile phase of 0.2 M ammonium acetate solution (pH 5) and acetonitrile 70:30 v/v) was eventually selected. Fig. 2 shows a chromatogram of a mixture of MLA and lappaconitine obtained using these conditions. MLA had a K' value of 6.5 and lappaconitine a K' value of 3.4, with a resolution of 2.4 even though the lappaconitine in particular showed tailing. The number of theoretical plates, N, for MLA was 3700 and 1700 for lappaconitine. The minor peak following MLA is nudicauline, which we have found as a minor contaminant in most of the MLA samples we have analysed.

3.2. Calibration

The calibration curve was linear from an MLA concentration of 2.8 to 100 μg ml^{-1} with a regression coefficient of 0.999. The line had a slope of 16.717 (SD = 0.136) and an intercept of 0.0268 (SD = 0.0055). The relative standard deviation (intra-day) at 5 μg ml^{-1} was 5% and 3.6% at 25 μg ml^{-1}. The lowest point on the calibration curve was equivalent to 280 ng of MLA on column. However, a peak for MLA was clearly detectable down to

approximately 50 ng on column, showing the potential for assaying down to this level by constructing appropriate calibration curves with a lower concentration of internal standard. Using a normal phase HPLC system with UV absorbance detection at 280 nm, Manners and Pfister [12] reported a detection limit for MLA of 300 ng on column. By using UV absorbance detection at 220 nm they were able to detect 30 ng on column, but this wavelength revealed that their chromatographic system did not fully resolve MLA from its parent alcohol, lycoctonine. This effectively prevented them from using this shorter wavelength for the quantitative analysis of MLA.

3.3. MLA content of alkaloids isolated from garden hybrid Delphinium seeds

Using UV detection at 270 nm, only those alkaloids possessing an aromatic acyl group were detected. However, LC/MS studies, to be reported elsewhere, showed that this system resolved MLA from all other alkaloids in the crude mixture. Using the assay described here the total crude alkaloid extracted from *Delphinium* seeds was shown to contain 56% (w/w) of MLA. The co-occurring alkaloid, delpheline, isolated from the crude mixture by vacuum liquid chromatography and recrystallisation, contained 0.2% (w/w) of MLA. Lycoctonine, prepared by base catalysed hydrolysis of MLA and purified by recrystallization contained no detectable MLA. The method can thus be applied both to the measurement of MLA in crude plant extracts and to isolated alkaloid samples in order to determine the precise levels of any trace contamination with this highly potent alkaloid prior to bioassay.

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Preliminary Synthetic Studies of Methyllycaconitine, a Potent Nicotinic Acetylcholine Receptor Antagonist: Rapid Syntheses of AE-Bicyclic Analogues

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Abstract

A series of bicyclic analogues incorporating the homocholine motif of methyllycaconitine has been prepared to test the hypothesis that this is the essential pharmacophore of this potent, selective nicotinic receptor antagonist. A double Mannich reaction has been employed to construct the 3-azabicyclo[3.3.1]-nonane ring system, containing an *N*-ethylpiperidine moiety. The neopentyl-like alcohol was then esterified, using isatoic anhydride under basic conditions, to afford the corresponding anthranilate.

There is a long history of the use of *Aconitum* and *Delphinium* by various civilisations as sources of poisons and medicines (Benn & Jacyno 1983). Probably the earliest use of a *Delphinium* preparation, as an insecticide, is the treatment of head and body lice reported by Pliny the Elder in AD 77. A pounded extract of *D. staphysagria* seeds was an efficient treatment for head and body vermin. Possibly significantly, the pounded flowers could be ingested, in wine, to counteract the poison of serpents. Serpent bites, in particular, were treated by applications of *D. staphysagria*.

Methyllycaconitine (MLA) (**1**) is a competitive nicotinic acetylcholine receptor (nAChR) antagonist. As such, it displaces the snake toxin α-bungarotoxin from its binding site on the pentameric ligand-gated ion channel protein receptor. Apparently, a similar *Delphinium* preparation was a standard issue in the British Army at the time of the battle of Waterloo (Benn & Jacyno 1983) and this practice is apparently still recommended for the treatment of lice! *Delphinium* plants are held responsible for more cattle deaths in North America than any other poisonous plant (Nambi Aiyar et al 1979; Keeler 1975). In 1938, Manske examined the aerial portion of *Delphinium brownii* Rydberg and established one of the alkaloids to be MLA (**1**), the 2-[2-(*S*)-methylsuccinimido]-benzoate ester of the norditerpenoid lycoctonine (**2**) (Fig. 1) (Manske 1938). This complex hexacycle has been reported in at least 30 *Delphinium* species. MLA produces mortality in a broad spectrum of insects (Jennings et al 1986), its toxicity resulting from its being a highly potent competitive nAChR antagonist (Nambi Aiyar et al 1979; Jennings et al 1986; Ward et al 1990). nAChR is the principal receptor in the insect central nervous system. Several subtypes of nAChR, in the vertebrate nervous system as well as at neuromuscular junctions, have recently been characterized by molecular biological techniques. MLA (**1**) shows higher affinity for neuronal α-bungarotoxin binding sites both in insects and vertebrates than for any other nAChR subtype (Jennings et al 1986; Ward et al 1990). In contrast, lycoctonine (**2**), the parent neopentyl-like alcohol, derived from MLA by saponification, exhibits markedly less nicotinic activity, indicating that, at least, the anthranilate moiety is significant in the structure-activity profile (Jennings et al 1986; Ward et al 1990).

The portion of the MLA molecule leading from the ester carbonyl function through carbon atoms 18, 4, and 19 to the *N*-ethyl group bears a formal resemblance to an acylated homocholine motif. In our structure-activity relationship (SAR) studies, we aim to explore the roles of this motif and the acylating anthranilic acid moiety in the MLA pharmacophore. We have, therefore, designed and synthesized a series of small molecule bicyclic analogues of MLA, as one part of our preliminary synthetic studies on this norditerpenoid alkaloid. These [3.3.1] bicycles have been designed to incorporate the 3-aminopropan-1-ol motif. In this communication, we present rapid and efficient syntheses of AE-bicyclic analogues which contain the piperidine (E) and cyclohexane (A) rings of MLA (**1**).

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Materials and Methods

General details

Thin layer chromatography was performed on pre-coated plates (Merck TLC aluminium sheets silica 60 F_{254}). The plates were visualized either with ninhydrin in *n*-butanol followed by heating with a hot air gun, or by short wavelength (254 nm) ultraviolet light. Column chromatography was performed according to the method developed by Still and co-workers (1978) using Sorbsil C60-H flash chromatography silica gel (40–60 μm) purchased from Prolabo, Eccles, Manchester. ^{1}H NMR spectra were recorded at 270 MHz using a Jeol GX270 spectrometer. ^{13}C NMR spectra were recorded at 67·8 MHz (GX270) employing 90

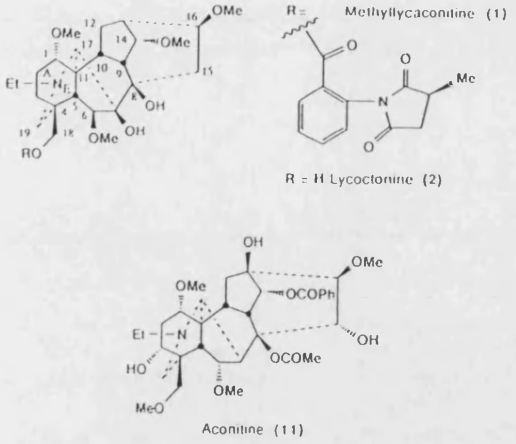

FIG. 1. Comparison of structures

and 135 DEPT pulse sequences to aid multiplicity determinations. Low resolution mass spectra were recorded on a VG Analytical 7070E with a VG 2000 data system. EI spectra were produced at 70 eV; CI was employed using *iso*-butane as the reagent gas, and +ve and −ve FAB was performed using 3-nitrobenzyl alcohol as the matrix. IR spectra were obtained using thin discs (KBr) on a Perkin-Elmer 782 infrared spectrophotometer and UV spectra were obtained in aqueous solution with a Perkin-Elmer Lambda 3 UV/Vis spectrophotometer. All chemicals and reagents were purchased from Aldrich. Solvents were purchased from Fisons and were either HPLC grade or were purified according to Perrin and Amarego (1988).

Results and Discussion

Synthesis of bicyclic alcohols 7 *and* 10 (Fig. 2)

The 9-substituted bicyclic alcohols (3-alkyl-3-aza-9-(*RS*)-methoxy- and methoxymethylbicyclo[3.3.1]nonane-1-methanols) 7 and 10 were designed to contain the AE ring system found in MLA (1). The synthesis of these bicycles, similar to those found in atisine (Ihara et al 1990), cardiopetaline (Shishido et al 1986), and related *Aconitum* and *Delphinium* alkaloids, was achieved in each case in four steps from ethyl 2-cyclohexanone-1-carboxylate (3), enabling subsequent conversion into the corresponding anthranilate esters (as seen in Fig. 2).

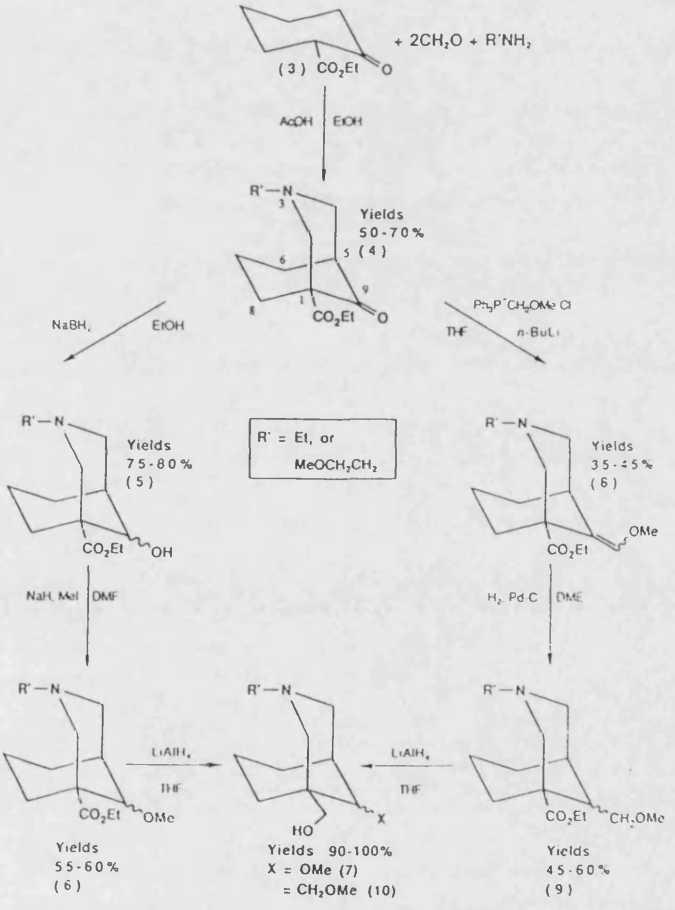

FIG. 2. Synthesis of bicyclic alcohols 7 and 10

Thus, a double Mannich condensation between keto-ester (**3**), a primary amine ($R'NH_2$), and aqueous formaldehyde (37%, 2 equivalents, reflux in ethanol in the presence of a catalytic amount of glacial acetic acid) was used to construct the 3-azabicyclo[3.3.1]nonane system (**4**), typical yields 50–70%. Both ethylamine and β-methoxyethylamine were used as the amine to give two parallel sets of analogues (Blicke & M^{c}Carty 1959). The basicity of a tertiary amine can be significantly reduced by the addition of a β-methoxyethyl group and therefore, it was hoped that this series would be more lipophilic at physiological pH (Perrin et al 1981). In one series of AE-bicyclic analogues, we decided to replace carbon C-6 (norditerpenoid numbering) with an *O*-methyl ether at C-5. Thus, reduction of **4** with sodium borohydride (carried out at room temperature in anhydrous ethanol) gave a mixture of epimeric alcohols (**5**), in 75–80% yields, which were then *O*-methylated (using sodium hydride and methyl iodide in anhydrous *N,N*-dimethylformamide) to give **6** in 55–60% yields. In another series of analogues, we extended the [3.3.1]bicycle carbon skeleton from the ketone at C-5 to C-6 (norditerpenoid numbering) by a Wittig reaction. Thus, the *E* and *Z* enol ethers (**8**) were synthesized from **4** by preparing the phosphorus ylid in-situ from *n*-butyllithium and (methoxymethyl)triphenyl phosphonium chloride in anhydrous tetrahydrofuran. Catalytic hydrogenation (5 atm) was then used to reduce the mixture of enol ethers (**8**) to the 9-(*RS*)-methoxymethyl ethers (**9**) using 10% palladium on charcoal in anhydrous 1,2-dimethoxyethane, typical yields 35–45%. Reduction of the ester functionality in **6** and **9** was achieved using lithium aluminium hydride in anhydrous tetrahydrofuran to give, in almost quantitative yields, the corresponding neopentyl-type alcohols **7** and **10**, respectively.

General procedure for synthesis of 2-(RS)-methylsuccinimidobenzoates (Fig. 3)

The efficient preparation of the intermediate 2-aminobenzoates was performed by heating isatoic anhydride, at 60°C, with the appropriate aliphatic alcohol in the presence of the basic catalyst, 4-(*N,N*-dimethylamino)pyridine in *N,N*-dimethylformamide (Perrin et al 1981), in 50–90% yields. Under these basic conditions, the formation of the ester is promoted over the carbamate. The conversion of these esters into their corresponding imides was achieved by fusion with two equivalents of 2-(*RS*)-methylsuccinic anhydride (Morrell 1914), typically for 2–4 h resulting in 40–70% yields of the isolated, desired 2-methylsuccinimidobenzoates.

It was found that at most stages in our synthetic routes only modest separation of the epimers was obtainable by chromatography over silica gel, but *O*-methyl ethers (**6**) were separable which enabled full characterization following from detailed inspection of their ^{1}H NMR spectra (COSY and nOe experiments). Further synthetic and SAR studies in this research area are ongoing in our laboratories. Other workers in this area recently reporting analogues of these complex, hexacyclic norditerpenoid alkaloids include the research groups of: Wiesner et al (1978), van der Baan et al (1992) Kraus et al (1993), and Whiting et al (1994).

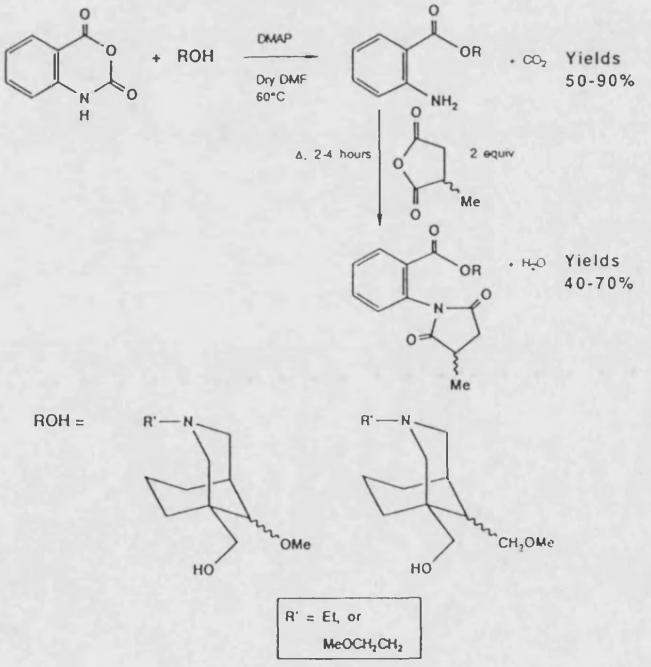

FIG. 3. General procedure for the synthesis of 2-(*RS*)-methylsuccinyimidobenzoates.

MLA (1) (free base) isolated in our studies, displayed comparable activity to authentic MLA citrate at both [^{125}I]α-bungarotoxin sites and [^{3}H]nicotine sites ($K_i = 2{\cdot}5 \pm 0{\cdot}9 \times 10^{-9}$ and $1{\cdot}3 \pm 0{\cdot}8 \times 10^{-5}$ M, respectively) (Coates et al 1994). Lycoctonine (2), produced by controlled hydrolysis of the ester in MLA, was four orders of magnitude less potent than MLA at the [^{125}I]α-bungarotoxin site ($K_i = 5{\cdot}0 \pm 1{\cdot}0 \times 10^{-5}$ M) (Coates et al 1994). Aconitine (11), a closely related neurotoxin from *Aconitum*, which interacts with sodium channels at the site characterized by batrachotoxin (Ward et al 1990), displayed poor activity ($K_i = 1{\cdot}9 \pm 0{\cdot}4 \times 10^{-5}$ and $>10^{-3}$ M, respectively). The striking difference in biological activity between MLA (1) and lycoctonine (2) however, indicates that the *N*-succinyl anthranilate ester moiety is significant.

MLA (1) is, therefore, a potent competitive nAChR antagonist and the first low molecular weight ligand able to discriminate between nAChR subtypes in vertebrates, preferring neuronal over neuromuscular. Our synthetic [3.3.1] bicyclic analogues of MLA, functionalized as their 2-methylsuccinimidobenzoate esters will allow us to test further the significance of the anthranilate moiety and the homocholine motif found around the AE bicycle of MLA (1).

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Appendix 2

Data to accompany the X-Ray Structure of Delpheline (219), Section 2.2.4 and Section 2.3.2.5 [Figure (8) shows the numbering scheme]:

Table I

Anisotropic Thermal Parameters U (Å^2 x10^3) for non-hydrogen atoms with estimated standard deviations (e.s.d.'s) in parentheses

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O1	40 (2)	52 (2)	80 (2)	20 (2)	-15 (2)	-12 (1)
O2	40 (1)	41 (1)	50 (2)	6 (1)	1 (1)	-8 (1)
O3	46 (2)	42 (1)	35 (1)	-2 (1)	-8 (1)	7 (1)
O4	37 (1)	45 (1)	45 (2)	-5 (1)	-9 (1)	5 (1)
O5	45 (2)	39 (1)	56 (2)	8 (1)	6 (1)	8 (1)
O6	46 (2)	31 (1)	86 (2)	16 (1)	6 (2)	0 (1)
N1	42 (2)	46 (2)	39 (2)	6 (2)	6 (2)	9 (2)
C1	41 (2)	48 (2)	38 (2)	8 (2)	-5 (2)	-2 (2)
C1'	48 (3)	54 (3)	99 (4)	-2 (3)	-16 (3)	-8 (2)
C2	42 (2)	57 (3)	53 (2)	11 (2)	-14 (2)	-1 (2)
C3	47 (2)	45 (2)	63 (3)	15 (2)	-3 (2)	10 (2)
C4	48 (2)	33 (2)	50 (2)	8 (2)	-5 (2)	4 (2)
C5	43 (2)	33 (2)	32 (2)	5 (2)	0 (2)	0 (2)
C6	37 (2)	30 (2)	41 (2)	-2 (2)	-6 (2)	-3 (2)
C7	35 (2)	38 (2)	29 (2)	-1 (2)	-3 (2)	0 (2)
C8	36 (2)	31 (2)	38 (2)	-1 (2)	-7 (2)	3 (2)

Table I cont.

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
C9	39 (2)	31 (2)	39 (2)	0 (2)	-1 (2)	3 (2)
C10	44 (2)	41 (2)	35 (2)	1 (2)	-7 (2)	1 (2)
C11	35 (2)	35 (2)	38 (2)	4 (2)	-1 (2)	0 (2)
C12	56 (3)	42 (2)	59 (3)	-6 (2)	-14 (2)	-4 (2)
C13	45 (2)	32 (2)	51 (2)	-3 (2)	-6 (2)	3 (2)
C14	51 (2)	35 (2)	39 (2)	-3 (2)	0 (2)	6 (2)
C14'	48 (2)	53 (3)	74 (3)	24 (2)	5 (2)	6 (2)
C15	47 (2)	39 (2)	47 (2)	4 (2)	0 (2)	3 (2)
C16	36 (2)	36 (2)	59 (3)	2 (2)	-1 (2)	4 (2)
C16'	52 (3)	99 (5)	392 (15)	138 (8)	43 (6)	6 (3)
C17	39 (2)	31 (2)	39 (2)	5 (2)	1 (2)	0 (2)
C18	57 (3)	36 (2)	77 (3)	8 (2)	-5 (3)	7 (2)
C19	48 (2)	37 (2)	48 (2)	4 (2)	-1 (2)	14 (2)
C20	44 (2)	47 (2)	46 (2)	-4 (2)	-8 (2)	2 (2)
C21	62 (3)	66 (3)	47 (2)	11 (2)	14 (2)	15 (3)
C22	71 (3)	80 (3)	66 (3)	10 (3)	21 (3)	14 (3)

The thermal parameter exponent takes the form:

$-2\,(U.h.a^* + ... + 2U.h.a^*.b^*)$

Table II

Final Fractional Atomic Co-ordinates ($x10^4$) and Equivalent Isotropic Thermal Parameters U_{eq} ($Å^2$ $x10^3$) for non-hydrogen atoms with e.s.d.'s in parentheses

Atom	x	y	z	U_{eq}
O1	-4868 (2)	5724 (2)	6150 (2)	57 (1)
O2	-551 (2)	7680 (2)	6533 (2)	44 (1)
O3	-1555 (2)	6763 (2)	8493 (2)	41 (1)
O4	-144 (2)	5987 (2)	7678 (2)	42 (1)
O5	53 (2)	4075 (2)	6327 (2)	46 (1)
O6	-1590 (2)	2935 (2)	7543 (2)	54 (1)
N1	-3910 (3)	7196 (2)	7766 (2)	42 (1)
C1	-4264 (3)	6577 (3)	5818 (3)	42 (1)
C1'	-5773 (4)	5452 (4)	5571 (4)	67 (2)
C2	-4984 (3)	7508 (3)	5941 (3)	51 (1)
C3	-4346 (4)	8485 (3)	5880 (3)	52 (1)
C4	-3363 (3)	8483 (3)	6556 (3)	44 (1)
C5	-2542 (3)	7644 (3)	6258 (2)	36 (1)
C6	-1594 (3)	7589 (3)	6961 (3)	36 (1)
C7	-1868 (3)	6625 (3)	7552 (2)	34 (1)
C8	-1207 (3)	5697 (3)	7283 (3)	35 (1)

Table II cont.

Atom	x	y	z	U_{eq}
C9	-1154 (3)	5608 (3)	6239 (3)	36 (1)
C10	-2348 (3)	5779 (3)	5830 (3)	40 (1)
C11	-3107 (3)	6593 (3)	6300 (3)	36 (1)
C12	-2849 (4)	4678 (3)	5796 (3)	52 (1)
C13	-1960 (3)	3974 (3)	6172 (3)	43 (1)
C14	-895 (3)	4531 (3)	5925 (3)	42 (1)
C14'	1056 (3)	4483 (3)	5992 (4)	58 (2)
C15	-1545 (3)	4707 (3)	7788 (3)	44 (1)
C16	-2088 (3)	3859 (3)	7235 (3)	44 (1)
C16'	-2190 (5)	2160 (5)	7689 (8)	181 (6)
C17	-3127 (3)	6482 (3)	7357 (3)	36 (1)
C18	-2815 (4)	9527 (3)	6506 (4)	56 (2)
C19	-3728 (3)	8265 (3)	7546 (3)	44 (1)
C20	-412 (3)	6475 (3)	8532 (3)	46 (1)
C21	-4102 (4)	7031 (4)	8756 (3)	58 (2)
C22	-5208 (4)	7378 (4)	9078 (3)	72 (2)

$U_{eq} = 1/3[U_{22} + 1/\sin^2\beta\ (U_{11} + U_{33} + 2U_{13}\cos\beta)] = 1/3$ trace $\hat{U}$,
where $\hat{U}$ is the diagonalized U_{ij} matrix.

Table III

Final Fractional Atomic Co-ordinates ($x10^4$) and Isotropic Thermal Parameters U ($Å^2$ $x10^3$) for hydrogen atoms with e.s.d.'s in parentheses

Atom	x	y	z	*U*
H(1,1)	-4103 (3)	6548 (3)	5171 (3)	66 (2)
H(1,1a)	-6147 (4)	4878 (4)	5827 (4)	66 (2)
H(1,1b)	-5501 (4)	5288 (4)	4968 (4)	66 (2)
H(1,1c)	-6281 (4)	6006 (4)	5528 (4)	66 (2)
H(2,1)	-5332 (3)	7474 (3)	6535 (3)	66 (2)
H(2,2)	-5542 (3)	7508 (3)	5470 (3)	66 (2)
H(3,1)	-4072 (4)	8568 (3)	5264 (3)	66 (2)
H(3,2)	-4832 (4)	9034 (3)	6029 (3)	66 (2)
H(5,1)	-2295 (3)	7800 (3)	5646 (2)	66 (2)
H(6,1)	-1538 (3)	8145 (3)	7382 (3)	66 (2)
H(9,1)	-601 (3)	6081 (3)	6042 (3)	66 (2)
H(10,1)	-2303 (3)	6096 (3)	5237 (3)	66 (2)
H(12,1)	-3029 (4)	4499 (3)	5173 (3)	66 (2)
H(12,2)	-3504 (4)	4641 (3)	6170 (3)	66 (2)
H(13,1)	-1989 (3)	3302 (3)	5928 (3)	66 (2)
H(14,1)	-698 (3)	4516 (3)	5285 (3)	66 (2)
H(14,a)	1670 (3)	4151 (3)	6283 (4)	66 (2)
H(14,b)	1083 (3)	5190 (3)	6127 (4)	66 (2)
H(14,c)	1097 (3)	4385 (3)	5337 (4)	66 (2)
H(15,1)	-2055 (3)	4892 (3)	8266 (3)	66 (2)
H(15,2)	-884 (3)	4434 (3)	8058 (3)	66 (2)
H(16,1)	-2874 (3)	3874 (3)	7341 (3)	66 (2)

Table III cont.

Atom	x	y	z	*U*
H(16,a)	-1731 (5)	1612 (5)	7890 (8)	66 (2)
H(16,b)	-2562 (5)	1975 (5)	7129 (8)	66 (2)
H(16,c)	-2730 (5)	2310 (5)	8155 (8)	66 (2)
H(17,1)	-3399 (3)	5867 (3)	7622 (3)	66 (2)
H(18,1)	-2196 (4)	9550 (3)	6921 (4)	66 (2)
H(18,2)	-3345 (4)	10034 (3)	6676 (4)	66 (2)
H(18,3)	-2563 (4)	9649 (3)	5889 (4)	66 (2)
H(19,1)	-4409 (3)	8619 (3)	7655 (3)	66 (2)
H(19,2)	-3165 (3)	8517 (3)	7953 (3)	66 (2)
H(20,1)	45 (3)	7062 (3)	8612 (3)	66 (2)
H(20,2)	-293 (3)	6021 (3)	9037 (3)	66 (2)
H(21,1)	-4040 (4)	6323 (4)	8880 (3)	66 (2)
H(21,2)	-3543 (4)	7388 (4)	9095 (3)	66 (2)
H(22,1)	-5279 (4)	7249 (4)	9726 (3)	66 (2)
H(22,2)	-5777 (4)	7022 (4)	8750 (3)	66 (2)
H(22,3)	-5280 (4)	8088 (4)	8966 (3)	66 (2)
H(O2)	-597 (38)	7681 (35)	5893 (13)	66 (2)

Key to symmetry operations relating designated atoms to reference atoms at (x, y, z):

(a) -x, -0.5+y, 1.5-z

Table IV

Bond Distances (Å) with e.s.d.'s in parentheses

C1-O1	1.430 (6)	C1'-O1	1.424 (6)
C6-O2	1.409 (6)	C7-O3	1.429 (5)
C20-O3	1.432 (6)	C8-O4	1.455 (5)
C20-O4	1.436 (6)	C14-O5	1.419 (5)
C14'-O5	1.412 (6)	C16-O6	1.438 (5)
C16'-O6	1.276 (7)	C17-N1	1.464 (6)
C19-N1	1.471 (6)	C21-N1	1.472 (6)
C2-C1	1.522 (8)	C11-C1	1.562 (7)
C3-C2	1.510 (8)	C4-C3	1.540 (8)
C5-C4	1.551 (7)	C18-C4	1.537 (8)
C19-C4	1.530 (8)	C6-C5	1.535 (7)
C11-C5	1.554 (7)	C7-C6	1.576 (7)
C8-C7	1.518 (7)	C17-C7	1.557 (7)
C9-C8	1.522 (7)	C15-C8	1.559 (7)
C10-C9	1.574 (7)	C14-C9	1.532 (7)
C11-C10	1.572 (7)	C12-C-C10	1.582 (8)
C17-C11	1.542 (7)	C13-C12	1.523 (8)
C14-C13	1.525 (8)	C16-C13	1.558 (8)
C16-C15	1.530 (7)	C22-C21	1.487 (7)

Table V

Bond Angles (°) with e.s.d.'s in parentheses

C1'-O1-C1	113.1 (4)	C20-O3-C7	105.0 (4)
C20-O4-C8	105.2 (4)	C14-O5-C14	122.7 (4)
C16'-O6-C16	120.2 (5)	C19-N1-C17	116.2 (4)
C21-N1-C17	113.6 (4)	C21-N1-C19	112.3 (4)
C2-C1-O1	108.2 (4)	C11-C1-O1	108.5 (4)
C11-C1-C2	116.5 (4)	C3-C2-C1	113.6 (4)
C4-C3-C2	110.7 (4)	C5-C4-C3	108.4 (4)
C18-C4-C3	107.4 (4)	C18-C4-C5	11.1 (4)
C19-C4-C3	112.1 (5)	C19-C4-C5	108.1 (4)
C19-C4-C18	109.8 (5)	C6-C5-C4	108.9 (4)
C11-C5-C4	110.7 (4)	C11-C5-C6	104.9 (4)
C5-C6-O2	111.6 (4)	C7-C6-O2	119.8 (4)
C7-C6-C5	104.1 (4)	C6-C7-O3	111.1 (4)
C8-C7-O3	102.2 (4)	C8-C7-C6	114.2 (4)
C17-C7-O3	116.6 (4)	C17-C7-C6	101.8 (4)
C17-C7-C8	111.6 (4)	C7-C8-O4	98.5 (4)

Table V cont.

C9-C8-O4	112.1 (4)	C9-C8-C7	109.9 (4)
C15-C8-O4	105.6 (4)	C15-C8-C7	155.2 (4)
C15-C8-C9	114.4 (4)	C10-C9-C8	109.0 (4)
C14-C9-C8	122.2 (4)	C14-C9-C10	102.0 (4)
C11-C10-C9	117.9 (4)	C12-C10-C9	103.2 (4)
C12-C10-C11	115.2 (4)	C5-C11-C1	112.8 (4)
C10-C11-C1	108.5 (4)	C10-C11-C5	110.2 (4)
C17-C11-C1	115.4 (4)	C17-C11-C5	97.6 (4)
C17-C11-C10	112.0 (4)	C13-C12-C10	106.7 (4)
C14-C13-C12	102.2 (4)	C16-C13-C12	110.2 (5)
C16-C13-C14	111.3 (4)	C9-C14-O5	116.1 (4)
C13-C14-O5	112.1 (4)	C13-C14-C9	102.2 (4)
C16-C15-C8	119.1 (4)	C13-C16-O6	110.5 (4)
C15-C16-O6	106.6 (4)	C15-C16-C13	113.9 (4)
C7-C17-N1	118.4 (4)	C11-C17-N1	110.6 (4)
C11-C17-C7	98.8 (4)	C4-C19-N1	115.4 (4)
O4-C20-O3	107.6 (4)	C22-C21-N1	113.7 (5)

Table VI

Selected Non-Bonded Distances (Å) (intramolecular and intermolecular)

D	H	A	D···A
N1	-	O1	3.265
O6	H(O2)	O2	2.930